Protein supplementation as a dietary strategy to improve glycemic control in type 2 diabetes

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The study presented in this thesis was performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM) which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

Cover design: Rob Schell
Printed by: Datawyse, Universitaire Pers Maastricht

ISBN 978-90-5278-761-9

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht
op gezag van de Rector Magnificus,
Prof. mr. G.P.M.F. Mols
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 14 november 2008 om 10:00 uur

door

Ralph Johannes Francisca Manders

geboren te Venray op 13 augustus 1978

UNIVERSITAIRE PERS MAASTRICHT

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The research described in this thesis was supported by a grant from DSM Food Specialties.

Financial support by the Dutch Diabetes Association for the publication of this thesis is gratefully acknowledged. Additional support was granted by DSM Food Specialties, Euregio Laboratory Sevices and A. Menarini Diagnostics Benelux.

Het duurt even, maar dan heb je ook wat.

Contents

Chapter 1

Introduction

Worldwide globalization and industrialization are accompanied by a more sedentary lifestyle and the consumption of an energy-rich diet, both of which are major contributors to the incidence of type 2 diabetes[1]. Over the past decades diabetes has claimed its place as one of the major threats to human health in the 21st century. Estimates of global diabetes prevalence predict an epidemic increase from 150 million people with diabetes in 2000, to 221 million in 2010 and as much as 366 million people worldwide by the year 2030[2, 3]. The Dutch National Institute for Public Health and the Environment (RIVM) reported that in 2003 approximately 700.000 people were diagnosed with diabetes and it is predicted that the prevalence of diabetes will increase with 32.5% from 2005 to 2025[4].

The primary treatment regime for type 2 diabetes is a pharmacological intervention with oral blood glucose lowering medication (i.e. insulin sensitizers or insulin secratogues) and, in a more advanced stage of the disease, exogenous insulin therapy or a combination of both. Apart from the pharmacological strategy, both nutritional and physical activity interventions can be implemented in the treatment of type 2 diabetes.[5-9]. This thesis will investigate the efficacy of proteins and amino acids as a dietary intervention to stimulate endogenous insulin release and improve glucose homeostasis in patients with type 2 diabetes.

Diabetes mellitus

Diabetes mellitus is a metabolic disease which is characterized by hyperglycemia (high blood glucose concentrations) as a result of insulin deficiency, reduced insulin sensitivity, or a combination of these factors. Long-term hyperglycemia can result in multi-organ failure, loss of vision and in extreme cases even amputation of a limb. The majority of all diabetes cases can be divided into two categories, i.e. type 1 and type 2 diabetes. Type 1 diabetes (or Insulin Dependent Diabetes Mellitus, IDDM) accounts for <10% of all diabetes cases[10]. Type 1 diabetes is primarily due to an auto-immune mediated destruction of the pancreatic β-cells resulting in a severe insulin secretory deficiency. Exogenous insulin replacement is currently the standard procedure for the treatment of type 1 diabetes. Type 2 diabetes (or Non-Insulin Dependent Diabetes Mellitus, NIDDM) which accounts for ~90% of all diabetes cases[10] is the result of peripheral insulin resistance and, in a later stage of the disease, insulin secretory deficiency. The remainder of diabetes cases (<5%) are the result of genetic defects of the β-cell (e.g. MODY or LADA), defects of insulin action (Leprechaunism) or disorders associated with impaired endocrine function (Cushing's syndrome).

Type 2 diabetes is normally diagnosed after the presentation of the classical diabetic symptoms (e.g. thirst, tiredness, glucosuria) and hyperglycemia. The clinically recommended procedure to diagnose diabetes is the Oral Glucose

Tolerance Test (OGTT)[11, 12]. After an overnight fast, a venous blood sample is drawn for the measurement of fasting plasma glucose concentrations. After the fasted sample is obtained, 75g of glucose (dissolved in 250 mL of water) is ingested and plasma glucose is determined again after a 2-hour period. The criteria for the diagnosis of diabetes and diabetes related conditions are presented in Table 1.1.

Table 1.1. Criteria for the diagnosis of diabetes and/or impaired glucose homeostasis†

	WHO/IDF	ADA
Diabetes		
Fasting plasma glucose	≥7.0 mmol/L	≥7.0 mmol/L
	(126 mg/dL)	(126 mg/dL)
2-h glucose	OR, ≥11.1 mmol/L	OR, ≥11.1 mmol/L
	(200 mg/dL)	(200 mg/dL)
Impaired Glucose Tolerant (IGT)		
Fasting plasma glucose	<7.0 mmol/L	Not required
	(126 mg/dL)	
2-h glucose	AND 7.8≤ X <11.0 mmol/L	7.8≤ X <11.0 mmol/L
	(140≤ X <200mg/dL)	(140≤ X <200mg/dL)
Impaired Fasting Glucose (IFG)		
Fasting plasma glucose	6.1≤ X ≤6.9 mmol/L	5.6≤ X ≤6.9 mmol/L
	(110≤ X ≤125 mg/dL)	(100≤ X ≤125 mg/dL)
2-h glucose	If measured, <7.8 mmol/L	If measured, <7.8 mmol/L
	(140 mg/dL)	(140 mg/dL)

† Comparison of diagnostic criteria as defined by the World Health Organization & the International Diabetes Federation[12] and the American Diabetes Association[11]. 2-h plasma glucose concentrations after ingestion of a 75g glucose load

Glucose homeostasis

Under normoglycemic conditions plasma glucose concentrations are tightly controlled by the pancreatic islet hormones, the liver and peripheral tissues (predominantly skeletal muscle and fat). Normal plasma glucose concentrations range from ~5.0 to <6.1 mmol/L in the overnight fasted state[12] up to approximately 10 mmol/L postprandially after consumption of a carbohydrate rich meal.

The pancreatic islets of Langerhans secrete the hormones insulin and glucagon which are responsible for controlling of the glucoregulatory feedback-loop. Insulin is a small globular protein which was first discovered in 1921[13] and which is produced by the β-cells of the pancreas. Insulin is able to lower circulating glucose concentrations by inhibiting hepatic glucose output and by stimulating glucose uptake in liver, skeletal muscle and adipose tissue. The

other major hormone involved in glucose homeostasis is glucagon. Glucagon was discovered shortly after the identification of insulin and is secreted by the α-cells of the islets of Langerhans[14]. The primary function of glucagon is to increase glucose concentrations during fasting or hypoglycemic episodes by stimulating hepatic glycogenolysis and gluconeogenesis.

Fasting glucose homeostasis

In the morning after an overnight fast, the liver is the major glucose producing organ. This is due to circulating glucagon levels which stimulate hepatic glucose production. The glucose produced by the liver is almost exclusively produced by glycogenolysis from stored liver glycogen and by gluconeogenesis using amino acids and glycerol as main carbon precursors. The glucose produced in the fasted state is predominantly taken up by insulin-insensitive tissues as the brain and the gut. Insulin sensitive tissues, like muscle and adipose tissue, take up relatively small amounts of glucose under overnight fasted conditions as fat is the major energy source in the fasted state. If blood glucose concentrations fall below a certain level (~5.0 mmol/L), glucagon secretion by the α-cells is stimulated and insulin secretion is inhibited, restoring normoglycemia.

Postprandial glucose homeostasis

As we generally consume at least three meals per day, and assuming that normal digestion by the gut takes ~5-6 hours[15], it is obvious that most humans are in a postprandial state throughout the greater part of the day. Therefore, postprandial blood glucose concentrations form the major determinant of 24 hour glycemia[16]. Following ingestion of a carbohydrate containing meal, blood glucose concentrations start to rise. As a result of this rapid increase in circulating blood glucose, hepatic glucose release can decline up to 80%[17]. Furthermore, β-cells are stimulated to secrete a rapid and transient burst of insulin from granules located near the plasma membrane. This first-phase insulin response increases circulating insulin concentrations up to 10-fold within minutes. However, this initial peak in insulin secretion is not maintained and subsides within 10 minutes to approximately 50% of the initial spike. Sustained hyperglycemia results in a second phase of stimulated insulin secretion. The second-phase insulin response consists of a slow but progressive increase in endogenous insulin secretion from more internally located insulin granules, and is continued for the duration of the elevated glucose levels[13].

The proportion of glucose contained in a meal that reaches the systemic circulation depends largely on splanchnic (hepatic and intestinal) extraction of glucose absorbed from the gut. Approximately 25% of the ingested glucose from a meal is disposed of in the splanchnic bed; the remainder is either oxidized or converted into glycogen and stored in skeletal muscle tissue[18]. A small portion of the ingested glucose (<5%) is stored as the glycerol moiety of

triacylglycerol in adipose tissue and/or the intramuscular or intrahepatic lipid pools.

Pathophysiology of hyperglycemia in type 2 diabetes

The two hallmark features in the pathogenesis of type 2 diabetes are insulin resistance and the inability of the pancreas to produce sufficient quantities of insulin to maintain normoglycemia. Insulin resistance is currently believed to be the first step in the development of type 2 diabetes and represents the inability of peripheral tissues (i.e. skeletal muscle and adipose tissue) to take up glucose under the influence of insulin[19]. With this emerging insulin resistance, the inhibitory effect of insulin on the liver decreases resulting in an increased hepatic glucose output. In the early stages of the development of the disease, the β-cells overcome the developing insulin resistance by overproducing insulin[20] (compensatory hyperinsulinemia) which generally results in normoglycemia. However, as insulin resistance prevails, the β-cells are no longer able to produce sufficient amounts of insulin to prevent hyperglycemia. This inability of the pancreas to secrete sufficient amounts of insulin has been suggested to be the result of β-cell failure[21].

The defects resulting in this β-cell failure include a reduced early insulin secretory response to oral glucose, a decline in the glucose-sensing ability of the β-cell, and a shift to the right in the dose-response curve relating glucose and insulin secretion, which are all indicative of a progressive insensitivity of the β-cell to glucose[20, 21].

In longstanding type 2 diabetes patients insulin secretion has been shown to be severely blunted following carbohydrate ingestion[22]. This reduced glucose induced insulin secretory response represents an important factor contributing to the elevated postprandial blood glucose excursions observed in these patients[22]. Epidemiological surveys and intervention studies have shown that postprandial hyperglycemia is both a direct and independent risk factor for the development of cardiovascular disease in type 2 diabetes[23-26]. Moreover, it has been reported that the rapid postprandial rise in blood glucose concentrations, also referred to as hyperglycemic spikes, are even more relevant to the onset of cardiovascular complications than merely elevated fasting blood glucose levels[27-29]. In accordance, both the Diabetes Control and Complications Trial (DCCT)[23] and the U.K. Prospective Diabetes Study (UKPDS)[24-26] report that improving glycemic control effectively reduces the risk of developing micro- and macrovascular complications and cardiovascular disease. Therefore, therapeutic strategies in the treatment of type 2 diabetes should focus on attenuating the postprandial rise in blood glucose concentrations.

Nutrition and type 2 diabetes

Nutritional recommendations can be used to fulfill several distinct roles in the treatment of type 2 diabetes. In healthy people or people at risk of developing diabetes (e.g. people with impaired glucose tolerance and/or obesity) diet can be modulated to prevent or delay the onset of diabetes. For patients who have been diagnosed with type 2 diabetes, nutritional modulation can be used to increase weight-loss and lower glucose concentrations. Dietary restriction has also been used successfully to treat or prevent early symptoms of the metabolic syndrome (obesity, dyslipidemia and hypertension) and diabetic complications such as cardiovascular disease, nephropathy and peripheral neuropathy.
Besides the 'classical' application of nutritional modulation (like energy intake restriction), there have been several recent suggestions that specific food-components (like amino acids and proteins) can be applied as so-called nutraceuticals to more directly modulate glycemic control.

Prevention of diabetes through dietary modulation

In recent years, several large intervention studies have been published that describe the clinical benefits of lifestyle intervention in the prevention of type 2 diabetes. Lifestyle interventions generally comprise of a combination of nutritional guidance (e.g. dietary modulation or restriction) and increased physical activity. As such, it is rather difficult to differentiate between the specific impact of dietary modulation and increased physical activity.
The Malmö prevention study[5] investigated the impact of diet and exercise over a 6 year period in males who were at risk for the development of type 2 diabetes. Lifestyle adaptation was shown to reduce the risk of diabetes by more than 50%. The Finnish Diabetes Prevention Study (DPS)[9] and the American Diabetes Prevention Program (DPP)[6], both providing a ~3 year lifestyle intervention with regular dietary guidance and exercise intervention reported that the risk for developing diabetes was reduced by 58% when compared to a control group not taking part in the program. An interesting finding from the DPP was the fact that treatment with metformin was able to reduce diabetes risk with 31%, implying that the lifestyle intervention was more effective in reducing the transition to type 2 diabetes than treatment with metformin[6]. In a Chinese study that differentiated between the impact of diet, exercise and the combination of the two, researchers reported that although diet was able to effectively reduce diabetes risk (-31%), exercise (-46%) and the combination of the two interventions (-42%) were able to further reduce the risk of developing type 2 diabetes[8]. In a preliminary analysis of the results after two years of lifestyle intervention, the Study on Lifestyle Intervention Maastricht (SLIM)[7] reported similar results. Although dietary intervention resulted in a reduced body weight and lowered 2-h plasma glucose concentration, the combination of diet and exercise showed the largest improvements in the appropriate parameters.

From all these studies, it is obvious that lifestyle intervention can have a compelling protective effect on the risk of developing type 2 diabetes.

Nutritional management of type 2 diabetes

Historically, nutritional recommendations for people with type 2 diabetes were simple and comprised basically of a single advice; to reduce the intake of sugar (carbohydrate). This advice was the direct consequence of the assumption that excursions in blood glucose concentrations were solely attributed to the consumption of carbohydrate. Current developments in the nutritional management of glycemia in type 2 diabetes generally focus on a healthy diet aimed at reducing diabetes related diseases, such as cardiovascular disease, by modulating the macronutrient composition of the diet. New trends in nutrition and dietetics in type 2 diabetes start to revert from the search for a standardized "Diabetes-Diet" to a more individualized approach to diet[30]. This individualization takes into account the changes (in lifestyle) the person is willing and able to make in order to achieve specific goals. Although this development could result in almost as many diets as there are type 2 diabetes patients, there are several goals and guidelines which should be used as cornerstones of any diet aimed at controlling diabetes.

These goals are:

- To obtain (near) normal glucose and lipoprotein concentrations;
- To treat or prevent diabetic complications;
- To ensure an adequate nutritional status.

In general, dietary guidelines for people with type 2 diabetes[31] do not differ distinctly from general dietary recommendations for standard healthy nutrition[32]. Analyses by the International Obesity Task Force (IOTF)[33], presented in the World Health Report 2002 indicated that approximately 58% of diabetes globally can be attributed to having a BMI above 21 kg/m^2. Consequently, weight loss should have priority in the treatment of overweight and obese, type 2 diabetes patients. Although data on the long-term effects of weight loss on type 2 diabetic complications are scarce, short-term studies have established the benefits of weight loss as a means to augment insulin sensitivity and improve blood glucose and lipid profiles[34-37].

Carbohydrate

The glycemic response to carbohydrate ingestion depends on a variety of factors, which include the amount and source of carbohydrate and the composition of the meal. Co-ingestion of protein, fat and dietary fiber as well as the use of different sources of carbohydrate (from monosacharides to starches) strongly impact the glycemic response. Incorporation of foods into the diet which result in a lowered glycemic response could therefore be a useful tool in

obtaining a tighter glycemic control in patients with type 2 diabetes. To better assess the glycemic response to a food Jenkins[38] introduced the concept of the Glycemic Index (GI). The glycemic index is the incremental area under the curve of the blood glucose produced by a standard amount of carbohydrate (normally 50g) in a food relative to the glycemic response of a reference food (usually white bread). There have been several longer-term studies investigating the possible benefits of low-glycemic index over high-glycemic index diets. However, these studies do not provide a clear picture as some studies[39-42] have reported positive effects of relatively low-glycemic index diets on blood HbA1c, fructosamine and plasma lipids, whereas others[43-46] failed to confirm such findings. Although there is currently not sufficient information on the effects of low-GI versus high-GI diets on glycemia, products with a low-GI should be promoted as they are rich in fiber and micronutrients.
In weight-maintaining diets for patients with type 2 diabetes it has furthermore been shown that when carbohydrates are removed from the diet and are replaced by monounsaturated fat postprandial glucose and triglyceride concentrations can be reduced[47, 48]. However, as carbohydrate-rich foods form an important source of fiber, micronutrients and energy, they should not be cut indiscriminately from the diet. An additional argument against very-low carbohydrate diets is the concern that when carbohydrate is replaced in ad libitum diets, the resulting increase in fat intake could stimulate unwanted weight gain and the development of insulin resistance[49-51].

Fat
In general, with respect to fat intake, recommendations for people with diabetes are the same as for individuals with a high cardiovascular risk profile[52]. It is recommended to lower the intake of saturated fat and LDL cholesterol[53] as patients with diabetes have a higher risk for the development of CVD[54]. In weight-maintaining diets plasma cholesterol concentrations have been shown to decrease when low saturated fat consumption was substituted with high carbohydrate or monounsaturated fat intake[48]. However, high carbohydrate diets are generally accompanied with greater postprandial glucose, insulin and triglyceride concentrations.

Protein
The dietary intake and need for protein for people with diabetes is similar to that of non-diabetic subjects and should average ~1.0-1.2 g/kg bw/day. However, in some cases hyperglycemia can contribute to an increase in total protein turnover[55, 56] or lead to an increased protein breakdown[57] in type 2 diabetes patients. In such cases, it would be advisable to increase dietary protein intake. It has also been suggested that dietary protein could induce hyperglycemia through gluconeogenic conversion of amino acids into glucose. This assumption has been rebutted in a series of studies showing that protein

intake does not increase plasma glucose concentrations[58, 59]. Dietary protein has also been associated with the development of nephropathy in individuals with diabetes[30], but there is currently no evidence to support the belief that diets with a protein content of up to 20 En% result in the development of diabetic nephropathy[52]. In contrast, a diet high in protein could help to augment satiety following food intake, thereby reducing total energy intake, leading to weight loss and improvements in insulin sensitivity. This hypothesis however, remains to be tested in type 2 diabetes patients.
When considering all the different nutritional modulations mentioned above it is apparent that there is no single "Diabetes-Diet" which is suitable, or applicable, to every type 2 diabetic. The shift from this single, unifying diet towards a more individualized approach to the nutritional management of type 2 diabetes is much more favorable as it allows for the development of diets which are tailored to the specific needs and abilities of a patient. Although these diets can be very different in their specific modulation, all diets should be aimed at obtaining a normal body weight, decreasing (postprandial) blood glucose concentrations, obtain normal lipoprotein concentrations and ensure a sufficient nutritional status.

Insulinotropic properties of the co-ingestion of protein and carbohydrate

Besides the proposed impact of high protein diets on energy intake, there is ample evidence to support the belief that the protein content of a meal modulates the postprandial insulin and glucose response[22, 59-62]. The stimulating effect of the combined intake of carbohydrate and protein on plasma insulin release was reported for the first time in the 1960s[63, 64], and has since been confirmed in healthy subjects[65] and type 2 diabetes patients[59, 61, 66]. Furthermore, intravenous infusion of free amino acids has been reported to increase insulin secretion[67-69].
In a series of studies, Floyd and co-workers[60, 67, 69-71] reported strong insulinotropic responses following the intravenous administration of various free amino acids. A strong synergistic stimulating effect on insulin release was observed when leucine and arginine were infused in combination with glucose[68]. Furthermore, numerous *in vitro* studies using primary pancreatic islet cells or β-cell lines have reported strong insulinotropic effects for arginine, leucine, isoleucine, alanine and phenylalanine[71-79].
The mechanisms by which these amino acids stimulate insulin secretion tend to be diverse and have not yet been fully elucidated[80]. In the presence of glucose, amino acids like arginine have been shown to stimulate insulin secretion by directly depolarizing the plasma membrane of the β-cell[72], which opens up voltage activated Ca^{2+} channels, leading to the influx of Ca^{2+} and subsequent insulin exocytosis[75, 80] (Figure 1.1). Other amino acids may modulate their

insulinotropic properties through activating Ca^{2+} channels by their co-transport with Na^{+} [75, 81]. Furthermore, intracellular catabolism of all metabolizable amino acids will increase the intracellular ATP/ADP ratio, thereby closing ATP-sensitive K^{+} channels, which can also lead to the depolarization of the plasma membrane[80, 82, 83].

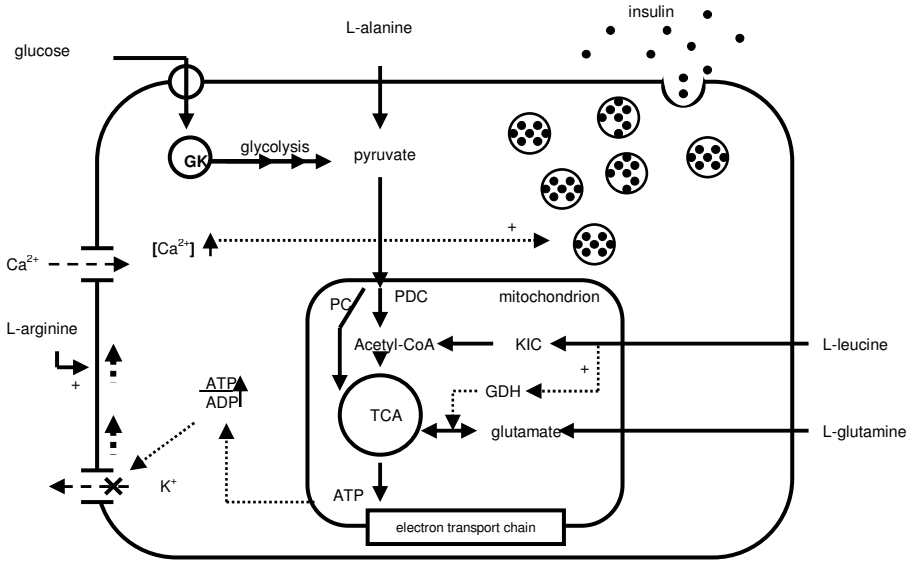

Figure 1.1. Amino acid induced insulin secretion. Adapted form Newsholme et al.[84]
GK, glucokinase; GDH, glutamate dehydrogenase; KIC, α-ketoisocaproate; PC, pyruvate carboxylase; PDC, pyruvate dehydrogenase complex; TCA, tricaboxylic acid cycle

Both *in vivo* and *in vitro* work has identified leucine as a particular interesting insulin secretagogue as it both induces and enhances pancreatic β-cell insulin secretion through its oxidative decarboxylation, as well as by its ability to allosterically activate glutamate dehydrogenase[78, 80, 85, 86] which increases ATP/ADP ratios by increasing TCA-cycle fluxes resulting in depolarization of the plasma membrane through closure of ATP-sensitive K^{+} channels. Furthermore, leucine can be transaminated to α-ketoisocaproate which in its turn is converted into acetyl-CoA before entering the TCA-cycle[87]. These findings are in line with recent *in vivo* observations, showing co-ingestion of relatively small amounts of free leucine to further augment the insulin response following the combined ingestion of carbohydrate and protein in healthy men[88]. Xu et al.[78] reported that the same signals that stimulate insulin release are also likely to be responsible for the leucine-induced activation of the mammalian target of rapamycin (mTOR) signaling pathway in the pancreatic β-cell. The potency of leucine to activate protein synthesis by interacting with the mTOR signaling pathway has been proposed to enhance β-cell function through the

maintenance of β-cell mass. As such, leucine administration has been suggested as an excellent candidate to optimize the insulinotropic effects of protein co-ingestion.

In young normoglycemic subjects, the ingestion of a protein hydrolysate/amino acid mixture with carbohydrate has been shown to represent a practical tool to accelerate post-exercise muscle glycogen synthesis by elevating insulin levels[89, 90]. In order to maximize the insulinotropic properties of protein co-ingestion, a mixture containing a protein hydrolysate and the free amino acids leucine and phenylalanine was defined to be most effective to stimulate endogenous insulin release[89, 91]. The insulinotropic properties of protein/amino acid mixtures could also be of clinical relevance in the treatment of type 2 diabetes. Increasing postprandial insulin secretion could accelerate blood glucose disposal thereby reducing postprandial hyperglycemia. However, as the insulin secretory response is severely reduced in longstanding type 2 diabetes patients, the application of insulinotropic amino acid/protein mixtures was questionable in this population. Therefore, van Loon et al.[22] studied the insulinotropic response to the co-ingestion of a protein hydrolysate/amino acid mixture with carbohydrate in longstanding type 2 diabetes patients in which compensatory hyperinsulinemia was no longer apparent. Co-ingestion of the amino acid/protein mixture was shown to substantially elevate the plasma insulin response 2-3 fold in these patients. These results imply that although the pancreatic β-cell response to glucose is severely impaired, the insulin secretory response to amino acids remains functional in longstanding type 2 diabetes patients.

The aim of this thesis is to further investigate the concept of protein/amino acid induced insulin secretion as a dietary strategy to stimulate endogenous insulin release and improve glucose homeostasis in type 2 diabetes patients.

Outline of this thesis

This thesis is comprised of a variety of human studies designed to investigate the potential of protein and amino acid co-ingestion as a dietary means to stimulate endogenous insulin release and improve glycemic control in longstanding, type 2 diabetes patients. **Chapter 2** studies the *de novo* insulin secretion following the combined ingestion of a mixture of protein hydrolysate, leucine and phenylalanine with carbohydrate in longstanding, type 2 diabetes patients and healthy, matched controls. A stable isotope glucose tracer was applied to determine whether greater endogenous insulin release improves postprandial blood glucose disposal. In **chapter 3** the postprandial plasma insulin, glucose and amino acid responses are studied following co-ingestion of an insulinotropic protein hydrolysate with and without additional free leucine with a single, meal-like, amount of carbohydrate. A continuous glucose monitoring system was used in **chapter 4** to assess, and compare glucose homeostasis in type 2 diabetes patients who receive standard medical care with the level of glycemic control in normoglycemic subjects under standardized dietary, but otherwise normal free living, conditions. The same glucose monitoring approach is applied in **chapters 5 & 6** to establish the clinical relevance and applicability of an insulinotropic protein hydrolysate with or without the addition of free leucine under real-life conditions. As the age-related loss of skeletal muscle mass is a major contributing factor associated with the development and progression of whole-body insulin resistance and type 2 diabetes, **chapter 7** investigates the effects of the combined ingestion of carbohydrate with protein on skeletal muscle protein synthesis in longstanding type 2 diabetes patients and healthy, matched controls. Finally, **chapter 8** combines the results and conclusions from the previous chapters and places them in a broader perspective. The applicability of protein and/or amino acid co-ingestion as a nutritional strategy to improve glucose homeostasis in type 2 diabetes patients will be discussed and future research aims will be addressed.

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Chapter 2

Co-ingestion of a protein hydrolysate/amino acid mixture with carbohydrate improves plasma glucose disposal in type 2 diabetes patients

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Am J Clin Nutr, 2005; 82(1): 76-83

Abstract

Objective: To investigate the insulin response and subsequent plasma glucose disposal rates following the ingestion of carbohydrate (CHO) or carbohydrate with a protein hydrolysate/amino acid mixture (CHO+PRO) in longstanding type 2 diabetes patients.

Design: Ten type 2 diabetes patients (age 62±2 y, BMI 27±1 kg/m^2) and nine healthy controls (age 58±1 y, BMI 27±1 kg/m^2) participated in 2 trials in which the plasma insulin response was determined following ingestion of 0.7 g/kg/h carbohydrate with or without 0.35 g/kg/h of a mixture containing a protein hydrolysate, leucine and phenylalanine. Continuous infusions with [6,6-$^2H_2$]glucose were applied to investigate plasma glucose disposal.

Results: Plasma insulin responses were increased by 299±64 and 132±63% in the CHO+PRO vs the CHO trial, in the diabetes patients and matched controls, respectively ($P<0.001$). The subsequent plasma glucose responses in the CHO+PRO trial were reduced by 28±6 and 33±3%, respectively, compared to the CHO trial ($P<0.001$). The latter was attributed to a 13±3% increase in postprandial glucose disposal ($P<0.01$).

Conclusion: The combined ingestion of carbohydrate with a protein hydrolysate/amino acid mixture substantially increases de novo insulin production in longstanding type 2 diabetes patients. This leads to increases in plasma glucose disposal and reduces postprandial glucose concentrations.

Introduction

The stimulating effect of the combined intake of carbohydrate and protein on plasma insulin release has already been reported in the 1960s[1, 2], and has since been confirmed in healthy subjects[3] and type 2 diabetes patients[4-6]. Furthermore, intravenous infusion of free amino acids has been reported to increase insulin secretion[7-9]. In line with these findings, various *in vitro* studies using incubated β-cells have attributed strong insulinotropic properties to arginine, leucine, and phenylalanine[10-17]. We have performed various *in vivo* studies in which we defined an optimal insulinotropic amino acid/protein mixture, containing leucine, phenylalanine and a protein hydrolysate, which has repeatedLy been shown to augment the insulin response by an additional 100% in healthy subjects[18, 19]. Nutritional interventions that effectively stimulate endogenous insulin secretion could be of particular significance in type 2 diabetes patients. Increasing endogenous insulin secretion could augment blood glucose disposal and, as such, improve glucose homeostasis. Moreover, preventing or reducing the postprandial rise in blood glucose concentration following carbohydrate intake could likely reduce the risk of developing diabetic and/or cardiovascular complications[20, 21]. Furthermore, the combined administration of amino acids/protein with carbohydrate, leading to a state of hyperinsulinemia and hyperaminoacidemia, may also represent an effective strategy to inhibit proteolysis and stimulate protein synthesis[22, 23]. The latter would also be of particular interest, as muscle protein breakdown rates are markedLy elevated in uncontrolled diabetes[24].

In longstanding type 2 diabetes patients, hyperglycaemia is no longer accompanied by a compensatory hyperinsulinemia. As such, it is generally assumed that the capacity of the β-cell to secrete insulin is severely impaired due to several defects[25]. These defects include a reduced early insulin secretory response to oral glucose, a reduced ability of the β-cell to compensate for the degree of insulin resistance, a decline in the glucose-sensing ability of the β-cell, and a shift to the right in the dose-response curve relating glucose and insulin secretion, which are all indicative of a progressive insensitivity of the β-cell to glucose[26]. All these defects involve glucose-sensing and -signalling pathways in the β-cell. Though insulin secretion in response to carbohydrate intake is impaired in type 2 diabetes patients, we recently showed that co-ingestion of a protein/amino acid mixture can increase the plasma insulin response 2-3 fold[27]. Though such nutritional interventions can effectively stimulate endogenous insulin secretion in longstanding type 2 diabetes patients, their clinical significance regarding blood glucose homeostasis remains to be established.

In the present study, we investigated the insulinotropic properties and the subsequent glucose disposal rate following the combined ingestion of a mixture of protein hydrolysate, leucine and phenylalanine with carbohydrate in longstanding type 2 diabetes patients and healthy, matched controls. Continuous infusions with [6,6-$^{2}H_{2}$]glucose were applied to determine plasma

glucose appearance and disappearance rates and, as such, to assess changes in glucose disposal. Our findings indicate that protein and/or amino acid co-ingestion represents an effective interventional strategy to elevate endogenous insulin secretion and to reduce the postprandial rise in blood glucose concentration following carbohydrate ingestion in longstanding type 2 diabetes patients.

Subjects and Methods

Subjects

Ten longstanding male type 2 diabetes patients and ten healthy, matched control subjects were selected to participate in this study. Subjects' characteristics are provided in Table 2.1. Exclusion criteria were impaired renal or liver function, obesity (BMI>35 kg/m^2), cardiac disease, hypertension, diabetes complications, and exogenous insulin therapy. Most type 2 diabetes patients (n= 9) were using oral anti-diabetic agents (metformin only or in combination with sulfonylureas); one subject did not use any medication. One control subject withdrew for the experiment for personal reasons. In the type 2 diabetes patients, blood glucose–lowering medication was withheld for 2 days prior to the screening and sulfonylureas were withheld 2 days before each of the trials. Subjects were screened for glucose intolerance/type 2 diabetes by a standard oral glucose tolerance test (OGTT) according to the World Health Organization criteria of 1999[28]. All subjects were informed about the nature and risks of the experimental procedures before their written informed consent was obtained. All clinical trials were approved by the local Medical Ethical Committee.

Screening

Before selection into the study, all subjects performed an OGTT. After an overnight fast, subjects arrived at the laboratory at 8.00 am by car or public transportation. A fasting blood sample was collected, after which a bolus of 75 g glucose (dissolved in 250 mL water) was ingested (t= 0 min). After 120 min a second blood sample was obtained. Plasma glucose concentrations were measured to determine glucose intolerance and/or type 2 diabetes according to the World Health Organization criteria of 1999[28]. In addition, basal fasting plasma glucose and insulin concentrations were used to assess whole-body insulin resistance using the homeostasis model assessment insulin resistance index (HOMA-IR)[29], which was calculated as the product of basal, fasting plasma glucose (mmol/L) and insulin (mU/L) concentrations divided by 22.5.

Table 2.1. Subject characteristics

	Controls	Type 2 diabetes
n	9	10
Age (y)	58.2±1.0	61.5±2.3
Body weight (kg)	84.89±2.86	81.8±3.89
Height (m)	1.76±0.02	1.73±0.02
BMI (kg/m^2)	27.49±1.07	27.19±0.97
Basal plasma glucose (mmol/L)	5.31±0.12	10.71±0.56 *
Plasma glucose$OGTT_{120}$ (mmol/L)	4.98±0.41	20.01±1.14 *#
Basal plasma insulin (mU/L)	6.44±0.90	10.30±1.59
HbA1c (%)	5.10±0.13	7.49±0.38 *
Homa-IR	1.52±0.22	5.02±0.96 *
Diagnosed with type 2 diabetes (y)	NA	11±2
Medication	NA	Metformin and/or SU-derivatives

Values are expressed as means±SEM. HOMA-IR homeostasis model assessment[29]. NA: not attained. *: significantly different from control group P<0.01; #: significantly different from basal values P<0.01.

Diet and activity prior to testing

All subjects maintained normal dietary and physical activity patterns throughout the entire experimental period. In addition, subjects refrained from heavy physical labour and exercise training for at least 3 days prior to each trial and filled out a food intake diary for 2 days prior to the first trial to keep their dietary intake as identical as possible prior to the other trial. The evening before each trial, subjects received a standardised meal (43.80 kJ/kg body weight; consisting of 60 Energy% (En%) carbohydrate, 28 En% fat and 12 En% protein).

Design

Each subject participated in two trials, separated by a 2 week period, in which the plasma insulin response and subsequent plasma glucose disposal rate were determined following the ingestion of 2 different beverage compositions (CHO; carbohydrate only or CHO+PRO; a mixture containing carbohydrate, a protein hydrolysate and the free amino acids leucine and phenylalanine) was determined. Subjects were placed in a supine position and remained inactive for a period of 3 hour. Drinks were provided in a randomized order and double blind fashion. Beverages were flavored to make the taste comparable in both trials.

Protocol

After an overnight fast, subjects reported to the laboratory at 08.00 am. A catheter (Baxter BV, Utrecht, the Netherlands) was inserted into an antecubital vein for isotope infusion. Another catheter was inserted into a dorsal vein on the contralateral hand, and was placed in a hot-box (60°C) for arterialized blood sampling. After 10 min, a resting blood sample was collected (t=0 min). After the administration of an intravenous 13.5 μmol/kg [6,6-$^2H_2$]glucose prime a continuous infusion of 277±3 nmol/kg/min of [6,6-$^2H_2$]glucose was started via a calibrated IVAC 560 pump (San Diego, CA, USA) and continued until t=180 min. At t=0 min subjects drank an initial bolus (2 mL/kg) of the test drink (CHO or CHO+PRO). Repeated boluses (2 mL/kg) were ingested every 15 min until t=165 min. Blood samples were drawn every 15 min during the first hour after which blood sampling occurred in 30 min intervals until t=180 min for measurement of plasma glucose, glucose-enrichment and insulin. In addition pro-insulin and C-peptide concentrations were measured in blood samples collected at t= 0, 60, 120, and 180 min.

Beverages

The subjects received repeated boluses of 2 mL/kg to ensure a given dose of 0.7 g/kg/h carbohydrate (50% glucose and 50% maltodextrin) with or without 0.35 g/kg/h of a protein hydrolysate/amino acid mixture (50% casein hydrolysate, 25% free leucine and 25% free phenylalanine) every 15 min until t=165 min. Glucose and maltodextrin were obtained from AVEBE (Veendam, the Netherlands), crystalline amino acids from BUFA (Uitgeest, the Netherlands), and the casein protein hydrolysate was prepared by DSM Food Specialties (Delft, the Netherlands). The casein hydrolysate was obtained by enzymatic hydrolysis of sodium caseinate using a neutral protease and a prolyl-specific endoproteinase (Insuvital™). Both drinks were uniformly flavored by adding 0.2 g sodiumsaccharinate, 1.8 g citric acid, and 5 g cream vanilla flavor (Quest International, Naarden, the Netherlands) per liter beverage.

Isotope tracer calculations

The glucose tracer (99% enriched, Cambridge Isotope laboratories, Andover, MA, USA) was first dissolved in 0.9% saline. Glucose tracer concentration in the infusates averaged 22±0.4 mmol/L. The [6,6-$^2H_2$]glucose infusion rate averaged 277±3 nmol/kg/min. Plasma glucose enrichments are expressed as tracer/tracee ratios (TTR). Rate of appearance (Ra) and rate of disappearance (Rd) of glucose were calculated using the single-pool non-steady state Steele equations[30] adapted for stable isotope methodology as described elsewhere[31].

$$Ra = \frac{F - V[(C_2 + C_1)/2]\ [(E_2 - E_1)/(t_2 - t_1)]}{(E_2 + E_1)/2} \quad (1)$$

$$Rd = Ra - V \cdot \left(\frac{C_2 - C_1}{t_2 - t_1} \right) \quad (2)$$

where F is the infusion rate (μmol/kg/min); V = distribution volume for glucose (160 mL/kg); C_1 and C_2 are the glucose concentrations (mmol/L) at time 1(t_1) and 2(t_2), respectively and E_2 and E_1 are the plasma glucose enrichments (TTR) at time 2 and 1, respectively.

Blood sample analysis

Blood (10 mL) was collected in EDTA containing tubes and centrifuged at 1,000 g and 4°C for 10 min. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at -80°C until analyses. Glucose concentrations (Uni Kit III, Roche, Basel) were analyzed with the COBAS FARA semi-automatic analyzer (Roche). Plasma insulin, pro-insulin and C-peptide was assayed with a modified, solid phase, two-site fluoroimmunometric assay based on a direct sandwich technique (DELFIA method, Perkin Elmer, Turku, Finland). To determine HbA1c content a 3 mL blood sample was collected in EDTA containing tubes and analysed by high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany). Following derivatisation, plasma [6,6-$^2H_2$]glucose enrichment was determined by electron ionisation GC-MS (Finnigan INCOS-XL).

Statistics

Data are expressed as means±SEM. The plasma responses were calculated as area under the curve minus baseline values. To compare plasma metabolite concentrations and tracer kinetics over time between trials, a two-way repeated measures analysis of variance (ANOVA) was applied. Changes in time within each group were checked for statistical significance using one-way repeated-measures ANOVA. A Scheffé's post-hoc test was applied in case of a significant F-ratio to locate specific differences. For non-time dependent variables, a multiway ANOVA or a Student's t-test for unpaired observations were applied. Significance was set at the 0.05 level of confidence. All calculations were performed using StatView 5.0 (SAS, Cary, NC, USA)

Results

Insulin

Fasting plasma insulin concentrations were similar in both groups and trials. Insulin concentrations increased significantly in both groups following the ingestion of carbohydrate and carbohydrate with the protein/amino acid mixture (P<0.001; Figure 2.1A). From t=60 min on, plasma insulin concentrations in the diabetes group were higher in the CHO+PRO trial compared to the CHO trial (P<0.05). No differences were found between trials in the control group. After expressing the insulin response as area under the curve (minus baseline values) significantly greater plasma insulin responses were observed in the CHO+PRO versus the CHO trial in both groups (P<0.01, Figure 2.1B). The plasma insulin response was 299±64 and 132±63% greater in the CHO+PRO vs the CHO trial, in the diabetes and matched control group, respectively (P<0.01).

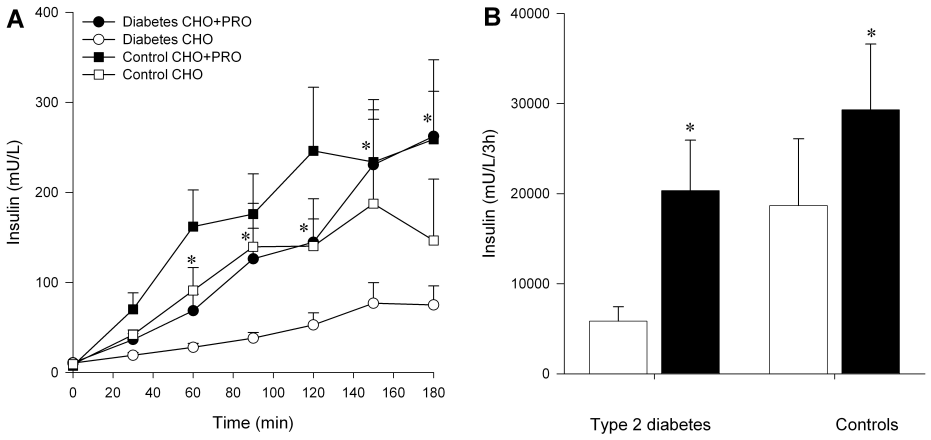

Figure 2.1. Plasma insulin concentrations (A) and responses (B) over a 3h period following the ingestion of carbohydrate (○/□, open bars) or carbohydrate and a protein hydrolysate/amino acid mixture (●/■, filled bars) in type 2 diabetes patients (○/●) and healthy, matched controls (□/■). Data are expressed as means ± SEM. *: Significantly different from CHO trial (P<0.05).

C-peptide and pro-insulin

Fasting plasma C-peptide concentrations were similar in both groups. In both trials C-peptide concentrations increased significantly over time (P<0.05; Figure 2.2A). From t=60 min on, plasma pro-insulin concentrations in the diabetes group were higher in the CHO+PRO trial compared to the CHO trial (P<0.05). When expressed as area under the curve significantly greater C-peptide responses were observed in the CHO+PRO vs the CHO trial in both groups (P<0.01). The plasma C-peptide responses were 98±18 and 56±26% greater in

the CHO+PRO vs the CHO trial, in the diabetes and control group, respectively (P<0.01). Plasma C-peptide concentrations correlated well with plasma insulin concentrations (r=0.89, P<0.001).

Fasting plasma pro-insulin concentrations were higher in the type 2 diabetes group compared to the normoglycemic controls (28.3±2.9 vs 7.5±0.5 mmol/L, respectively P<0.01). In both trials, pro-insulin concentrations increased significantly over time (P<0.01; Figure 2.2B). From t=120 min on, plasma pro-insulin concentrations in the diabetes group were higher in the CHO+PRO trial compared to the CHO trial (P<0.05). No differences were observed between trials in the control group. When expressed as area under the curve significantly greater pro-insulin responses were observed in the CHO+PRO vs the CHO trials in both groups (P<0.05). The plasma pro-insulin responses were 151±28 and 84±37% greater in the CHO+PRO vs the CHO trial, in the diabetes and matched control group, respectively (P<0.05). Plasma pro-insulin concentrations correlated with both plasma insulin and C-peptide concentrations (r=0.79 and r=0.85, respectively; P<0.001)

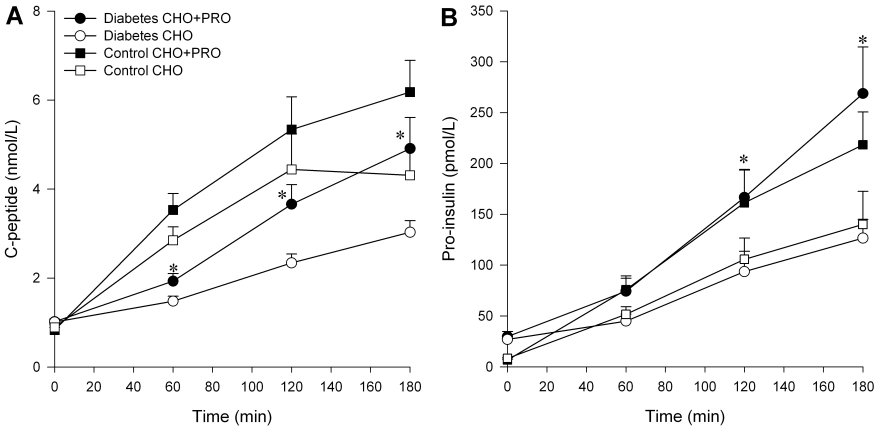

Figure 2.2. Plasma C-peptide (A) and pro-insulin concentrations (B) over a 3h period following the ingestion of carbohydrate (○/□, open bars) or carbohydrate and a protein hydrolysate/amino acid mixture (●/■, filled bars) in type 2 diabetes patients (○/●) and healthy, matched controls (□/■). Data are expressed as means ± SEM. *: Significantly different from CHO trial (P<0.05).

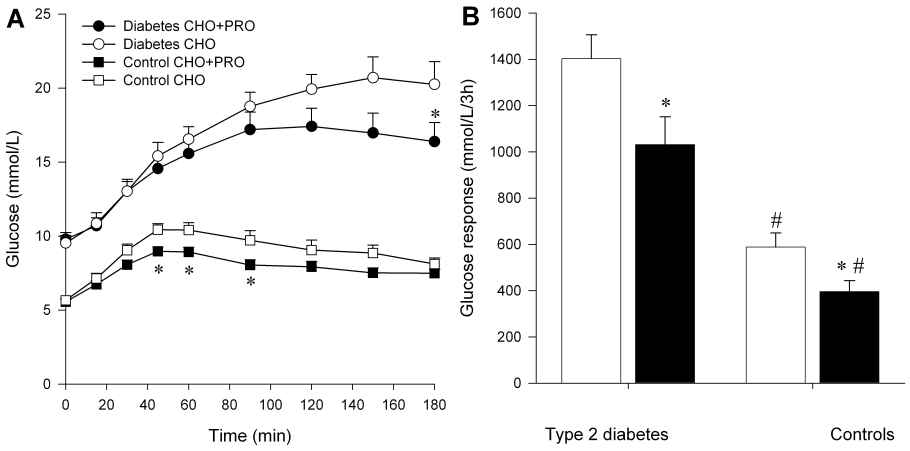

Figure 2.3. Plasma glucose concentrations (A) and responses (B) over a 3h period following the ingestion of carbohydrate (○/□, open bars) or carbohydrate and a protein hydrolysate/amino acid mixture (●/■, filled bars) in type 2 diabetes patients (○/●) and healthy, matched controls (□/■). Data are expressed as means ± SEM. *: Significantly different from CHO trial (P<0.05); #: significant difference between groups (P<0.001).

Glucose

Fasting plasma glucose concentrations were higher in the type 2 diabetes patients compared to their normoglycemic controls (9.7±0.3 vs 5.7±0.1 mmol/L, respectively P<0.01). In the type 2 diabetes patients, plasma glucose concentrations in the CHO trial increased after carbohydrate ingestion until t=150 min, after which values plateaued. In the CHO+PRO trial, glucose concentrations increased significantly (P<0.01) during the first 90 min, after which they plateaued or tended to decline (Figure 2.3A). At t=180 min plasma glucose concentration was significantly lower in the CHO+PRO trial vs the CHO trial (P<0.05) for the type 2 diabetes subjects. In the control group, plasma glucose concentrations slightly increased during the first 60 min in both trials and then returned to baseline levels over the next 2 h (Figure 2.3A). Plasma glucose concentrations were significantly higher in the type 2 diabetes patients compared to their matched controls (P<0.05). After expressing the plasma glucose response as area under the curve, we observed a significantly higher plasma glucose response in the type 2 diabetes patients vs their matched normoglycemic controls (P<0.001; Figure 2.3B). In both groups, significantly lower plasma glucose responses were observed in the CHO+PRO vs the CHO trial (P<0.001; Figure 2.3B). The plasma glucose response was 28±6 and 33±3% lower in the CHO+PRO vs the CHO trial, in the diabetes and matched control group, respectively (P<0.001).

Glucose tracer kinetics

In the type 2 diabetes group, plasma glucose Ra was stable over the entire period and averaged 42.4±0.8 and 41.2±1.1 μmol/kg/min in the CHO and CHO+PRO trial, respectively (NS). In the control group, plasma glucose Ra was also stable and averaged 39.8±0.7 and 37.9±0.8 μmol/kg/min in the CHO and CHO+PRO trial, respectively (Table 2.2 and Figure 2.4A and B). No significant differences in plasma glucose rate of appearance were observed between trials or groups.

Glucose Rd increased over time in both trials in both groups ($P<0.05$; Figure 2.4C and D). In the diabetes group, Rd averaged 19.7±2.4 and 20.4±2.8 μmol/kg/min at t=30 min and increased over time to reach 45.1±1.8 and 45.4±3.6 μmol/kg/min in the CHO and CHO+PRO trial, respectively (Figure 2.4C). In the control group, Rd averaged 14.7±1.4 and 19.4±1.7 μmol/kg/min at t=30 min and increased to 45.4±2.4 and 44.8±2.2 μmol/kg/min in the CHO and CHO+PRO trial, respectively (Figure 2.4D). The increase in Rd over time was significantly different between groups ($P<0.05$).

Plasma glucose disposal, expressed as the percentage of the appearing glucose that disappears from the circulation, was significantly lower in the diabetes patients compared to the matched controls ($P<0.001$; Figure 2.5). In the diabetes group the CHO+PRO trial resulted in a significant 12.5±3.1% increase in plasma glucose disposal compared to the CHO trial ($P<0.01$). In the control group, plasma glucose disposal was not significantly improved in the CHO+PRO trial (3.4±2.2%; $P=0.2$; Figure 2.5).

Table 2.2. Plasma glucose kinetics

	CHO	CHO+PRO
Controls		
Ra (μmol/kg/min)	39.76±0.73	37.89±0.79
Rd (μmol/kg/min)	36.23±1.69	35.68±1.32
Rd as% of Ra	90.84±3.97	93.58±2.76
Time for Rd to match Ra (min)	90±8	75±6*
Type 2 diabetes		
Ra (μmol/kg/min)	42.36±0.80	41.23±1.10
Rd (μmol/kg/min)	30.32±1.30	33.18±1.50
Rd as% of Ra	72.45±3.08#	81.02±3.11*#
Time for Rd to match Ra (min)	179±8#	135±9*#

Mean [6,6-$^2H_2$] glucose tracer rate of appearance (Ra) and rate of disappearance (Rd) and Rd expressed as percentage of Ra over the entire 150 min period. Values are expressed as means±SEM. *: significantly different from CHO trial $P<0.01$; #: significantly different from control group $P<0.01$.

In the diabetes group, the CHO+PRO trial improved glucose disposal rate by 15.8 g (i.e. ~88 mmol) over the 150 min period when compared to the CHO trial (P<0.01). In the control group an additional 11.7 g (~65 mmol) glucose was disposed of during the 150 min period when comparing the CHO+PRO to the CHO trial (P=0.2).

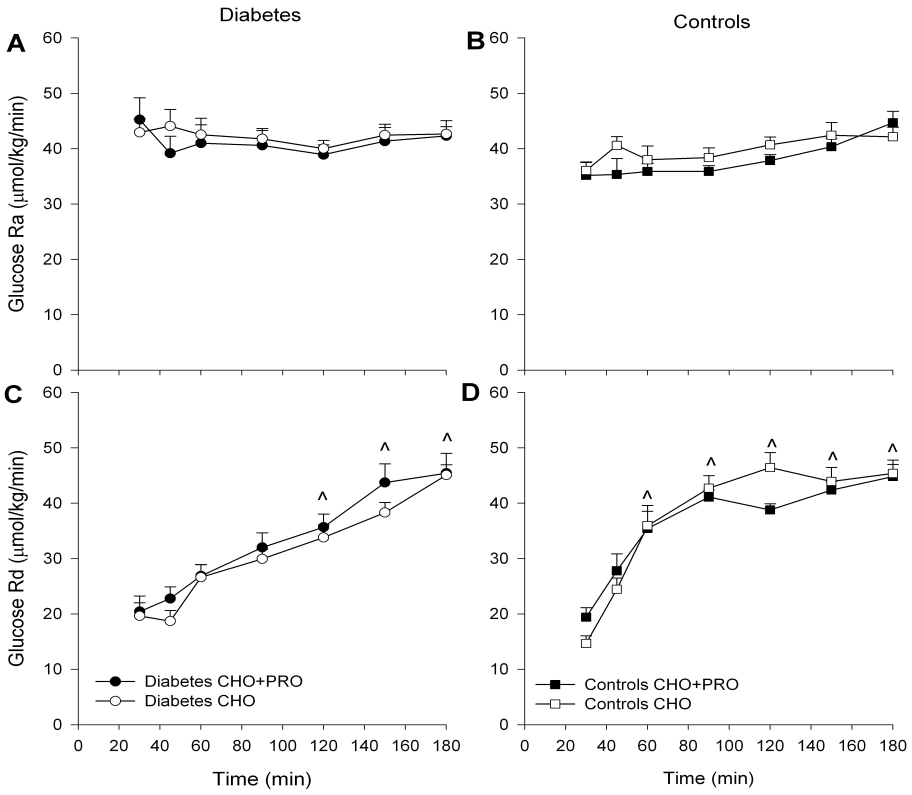

Figure 2.4. Plasma glucose rate of appearance (A/B) and disappearance (C/D) over time in type 2 diabetes patients (A/C) and healthy, matched control subjects (B/D) following the ingestion of carbohydrate (○/□) or carbohydrate and a protein hydrolysate/amino acid mixture (●/■) in type 2 diabetes patients (○/●) and healthy controls (□/■). Data are expressed as means ± SEM. ^: Significantly different from t=30 min (P<0.05)

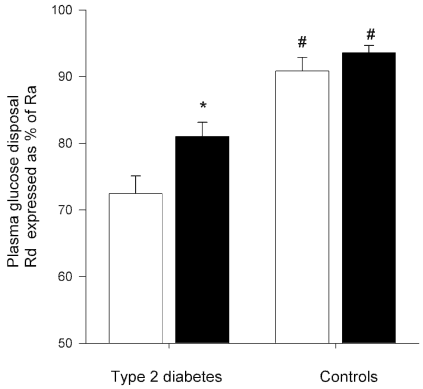

Figure 2.5. Plasma glucose disposal defined as plasma glucose rate of disappearance (Rd) expressed as a percentage of the appearance rate (Ra) following the repeated ingestion of carbohydrate (open bars) or carbohydrate and a protein hydrolysate/amino acid mixture (filled bars). Data are expressed as means ± SEM. *: Significantly different from CHO trial (P<0.001); #: significantly different from diabetes patients (P<0.001).

Discussion

The present study shows that co-ingestion of a mixture containing casein hydrolysate, leucine and phenylalanine substantially increases insulin secretion when compared to the ingestion of carbohydrate only. The substantial 3-4-fold greater insulin response significantly improves postprandial glucose disposal, resulting in lower plasma glucose concentrations in type 2 diabetes patients. This study indicates that nutritional interventions that improve endogenous insulin secretion can provide a practical and effective tool in the treatment of type 2 diabetes.

The synergistically stimulating effect of the combined ingestion of carbohydrate and intact protein on plasma insulin release was first reported in the late 1960's[1, 2], and later confirmed in healthy subjects[3] as well as type 2 diabetes patients[4-6]. Floyd et al.[7-9, 32] investigated the effects of intravenous infusion of various amino acids on plasma insulin secretion and reported that arginine, leucine and/or phenylalanine were most insulinotropic. Over the last few years we have confirmed many of their findings following oral administration of these amino acids in combination with carbohydrate[18, 19]. Consequently, we defined a practical and optimal insulinotropic amino acid/protein (hydrolysate) mixture containing a protein hydrolysate, free leucine and phenylalanine[18, 19]. Recently, we investigated the insulinotropic properties of this mixture in longstanding type 2 diabetes patients, and reported a 189% greater plasma insulin response when co-ingesting this mixture with carbohydrate[27]. Though that study clearly showed that endogenous insulin secretion can be substantially augmented in longstanding type 2 diabetes patients, it has not yet been established whether these findings are of clinical relevance. Therefore, in

the present study we investigated plasma glucose disposal following ingestion of carbohydrate with or without the addition of such an insulinotropic protein hydrolysate/amino acid mixture in healthy subjects and type 2 diabetes patients.

The type 2 diabetes patients selected in this study, had been diagnosed with type 2 diabetes for over 10 yrs. Basal fasting glucose concentrations, OGTT values, HbA1c content, and the HOMA-IR index values confirmed their type 2 diabetic state (Table 2.1). Hyperinsulinemia, as a compensatory response to the prevailing hyperglycemia, was no longer present in these longstanding type 2 diabetes patients (Table 2.1 and Figure 2.1). After ingestion of only carbohydrate in the CHO trial, insulin responses were shown to be substantially lower in the diabetes patients compared to their controls (Figure 2.1B). The latter clearly demonstrates the reduced sensitivity of the β-cell to glucose in the type 2 diabetic state[26]. Interestingly, co-ingestion of the protein hydrolysate/amino acid mixture in the CHO+PRO trial significantly increased the plasma insulin response by an additional 299±64 and 132±63% in the diabetes patients and their normoglycemic controls, respectively ($P<0.01$, Figure 2.1B). The insulin response in the CHO+PRO trial in the type 2 diabetes patients was similar to the insulin response reported in the CHO trial in the healthy subjects (Figure 2.1B). In other words, though the sensitivity of the pancreas to carbohydrate intake is significantly reduced in longstanding type 2 diabetes patients, the capacity to secrete insulin in response to other stimuli (like amino acids) has remained intact. Therefore, the defects in the insulin response to meal ingestion in these patients is mainly attributed to the reduced sensitivity of the β-cell to glucose, and not an overall defect in the capacity to produce and/or secrete insulin.

To confirm that the elevated plasma insulin concentrations in the CHO+PRO trial are indeed secondary to increased insulin production, we also determined plasma C-peptide and pro-insulin concentrations[33]. In the process of insulin production the precursor pro-insulin is cleaved into insulin and the 31-kD residue connecting peptide (C-peptide). Insulin, C-peptide and a small amount of residual pro-insulin are stored in the secretory granules of the β-cell until secretion[34]. In the present study, we observed a significant increase in plasma C-peptide and pro-insulin concentrations over time in all trials (Figure 2.2). Significantly greater plasma C-peptide responses were observed in the CHO+PRO vs the CHO trial (+98±18 and 56±26% in the diabetes patients and healthy controls, respectively; $P<0.01$). In accordance, plasma pro-insulin responses were also 151±28 and 84±37% greater in the CHO+PRO vs the CHO trial, in the diabetes and control group, respectively ($P<0.05$). Both C-peptide and pro-insulin concentrations correlated well with plasma insulin concentrations ($r=0.89$ and $r=0.79$, respectively: $P<0.001$). As such, these data further support the observation that co-ingestion of the protein/amino acid

mixture in the CHO+PRO trial effectively stimulates de novo insulin production.

In response to the increased insulin production and secretion rate in the CHO+PRO trial, plasma glucose concentrations were significantly reduced when compared to values observed in the CHO trial (Figure 2.3A). In the CHO+PRO trial, plasma glucose responses were decreased by as much as 28±6 and 33±3% in the diabetes patients and normoglycemic controls, respectively ($P<0.001$). This decline in plasma glucose response is much more prominent compared to our earlier observations[27], which is explained by the longer trial duration that was implemented in the present study. Interventions that effectively reduce the postprandial rise in plasma glucose concentrations following carbohydrate intake are of clinical significance, and have been associated with a reduced risk of developing diabetic and/or cardiovascular complications[20, 21]. Many food components or pharmacological agents, that have been shown to effectively lower postprandial glucose concentration after meal ingestion, inhibit gastric emptying and/or intestinal uptake of glucose[35-37]. In the present study, we applied a continuous infusion with a [6,6-$^2H_2$]glucose tracer to determine the appearance rate of glucose in the circulation. Plasma glucose appearance rates (Ra) were similar in both groups and trials and remained constant throughout the trials (Table 2.2, Figure 2.4A/B). The latter indicates that inhibition of gastrointestinal uptake of glucose is not responsible for the observed decline in the postprandial blood glucose response following co-ingestion of the protein/amino acid mixture.

Whereas, plasma glucose Ra remained stable throughout the trials, plasma glucose rate of disappeance from the circulation (Rd) significantly increased over time in both trials (Figure 2.4; $P<0.01$). In contrast to the Ra values, plasma glucose Rd was strikingly different between the diabetes patients and the healthy, matched controls (Figure 2.4C/D). Whereas in the controls Rd values increased exponentially, a more gradual rise in glucose Rd was observed in the diabetes patients ($P<0.01$). Consequently, it took the diabetes patients about twice as long before plasma glucose Ra was matched by its Rd ($P<0.01$). Consequently, plasma glucose disposal (calculated as Rd expressed as a percentage of Ra) was significantly impaired in the type 2 diabetes patients compared to the normoglycemic controls (Table 2.2 and Figure 2.5; $P<0.01$). In both groups the time for Rd to match Ra was significantly reduced in the CHO+PRO trial (Table 2.2: $P<0.01$). In accordance, plasma glucose disposal following co-ingestion of the protein/amino acid mixture was shown to improve plasma glucose disposal by 13±3% ($P<0.01$) and 3±2% ($P=0.2$) in diabetes patients and healthy controls, respectively.

In conclusion, co-ingestion of a protein hydrolysate, leucine and phenylalanine mixture can substantially augment the insulin response following carbohydrate intake. In longstanding type 2 diabetes patients, co-ingestion of such a mixture can induce a 3-4-fold greater plasma insulin response. The latter effectively

improves plasma glucose disposal, thereby reducing postprandial plasma glucose concentration. The combined ingestion of an amino acid and/or protein mixture with carbohydrate represents an effective interventional strategy in the treatment of type 2 diabetes.

Acknowledgements
We gratefully acknowledge the expert analytical assistance of Jos Stegen, Joan Senden and Annemie Gijsen as well as the enthusiastic support of the subjects who volunteered to participate in these trials. The described work was supported by a grant from DSM Food Specialties (Delft, The Netherlands).

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Chapter 3

Co-ingestion of a protein hydrolysate with or without additional leucine effectively reduces postprandial blood glucose excursions in type 2 diabetic men

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J Nutr, 2006; 136(5): 76-83

Abstract

Objective: This study examined the postprandial plasma insulin and glucose responses following co-ingestion of an insulinotropic protein hydrolysate with and without additional free leucine with a single bolus of carbohydrate.
Design: Ten longstanding male type 2 diabetes patients and ten healthy controls participated in 3 trials in which plasma glucose, insulin and amino acid responses were determined following the ingestion of beverages of different composition (CHO: 0.7 g/kg carbohydrate, CHO+PRO: 0.7 g/kg carbohydrate with 0.3 g/kg protein hydrolysate, or CHO+PRO+LEU: 0.7 g/kg carbohydrate, 0.3 g/kg protein hydrolysate and 0.1 g/kg free leucine).
Results: Plasma insulin responses (expressed as AUC) were 141 and 204% greater in the type 2 diabetes patients and 66 and 221% greater in the controls in the CHO+PRO and CHO+PRO+LEU trials respectively, when compared to the CHO trial ($P<0.05$). The concomitant plasma glucose responses were 15 and 12% lower in the type 2 diabetes patients and 92 and 97% lower in the control group in the CHO+PRO and CHO+PRO+LEU trials respectively, when compared to the CHO trial ($P<0.05$). Plasma leucine concentrations correlated with the insulin response in all subjects ($r=0.43$, $P<0.001$).
Conclusion: We conclude that co-ingestion of a protein hydrolysate with or without additional free leucine strongly augments the insulin response following ingestion of a single bolus of carbohydrate, thereby significantly reducing postprandial blood glucose excursions in longstanding type 2 diabetes patients.

Introduction

Many studies have reported the stimulating effect of the combined ingestion of carbohydrate and protein on insulin release *in vivo* in humans[1, 2]. In addition, strong insulinotropic responses have been reported following intravenous administration of various free amino acids[3-6]. Leucine has been identified as a particularly interesting insulin secretagogue as it both induces and enhances pancreatic β-cell insulin secretion through its oxidative decarboxylation, and by its ability to allosterically activate glutamate dehydrogenase[7-10]. In accordance, we have shown that a mixture containing a protein hydrolysate with additional free leucine and/or phenylalanine has strong insulinotropic properties in humans. In healthy males, co-ingestion of this mixture with carbohydrate augments the insulin response 2-3 fold when compared to the ingestion of only carbohydrate[11, 12].

In longstanding type 2 diabetes patients, hyperglycemia is no longer accompanied by compensatory hyperinsulinemia. Therefore, it is generally assumed that the absolute insulin secreting capacity of the pancreatic β-cell is substantially impaired in these individuals[13, 14]. As a consequence, it has been questioned whether amino acid induced insulin secretion could represent an effective strategy to improve blood glucose homeostasis in type 2 diabetes[11, 12]. We recently showed that the insulin response following continuous ingestion of large amounts of carbohydrate can be increased ~2-4 fold in longstanding type 2 diabetes patients, simply by co-ingesting a protein hydrolysate, leucine and phenylalanine mixture[15, 16]. Furthermore, we showed that the greater endogenous insulin response following protein/amino acid co-ingestion is accompanied by an increase in blood glucose disposal rate, resulting in a ~30% lower blood glucose response when compared to the ingestion of only carbohydrate[16]. Even though these proof-of-principle studies imply that protein and/or amino acid co-ingestion represents a promising strategy to improve blood glucose homeostasis in type 2 diabetes, it should be noted that these and other findings[11, 12, 15-18] have all been obtained in a setting in which excessive amounts of carbohydrate were continuously administered. It remains to be established whether co-ingestion of a protein and/or leucine mixture can lower postprandial blood glucose responses following the ingestion of a single, meal-like amount of carbohydrate.

In the present study, we determined the postprandial insulin, glucose and amino acid responses following the ingestion of a single bolus of carbohydrate with or without the addition of a protein hydrolysate or a protein hydrolysate/leucine mixture in both longstanding type 2 diabetes patients and healthy, normoglycemic controls.

Subjects and methods

Subjects

Ten longstanding male type 2 diabetes patients and ten healthy matched control subjects participated in this study (Table 3.1). Exclusion criteria were impaired renal or liver function, obesity (BMI>35 kg/m^2), cardiac disease, hypertension, diabetes complications, and exogenous insulin therapy. All type 2 diabetes patients were using metformin only (n=3), or metformin in combination with sulfonylureas (n=7). Blood glucose–lowering medication was withheld for 2 d prior to the screening and sulfonylureas were withheld 2 d before each trial. All subjects were informed about the nature and the risks of the experimental procedures before their written informed consent was obtained. All clinical trials were approved by the Medical Ethical Committee of the Academic Hospital of Maastricht.

Table 3.1. Subjects' characteristics

	Controls	Type 2 diabetes
Age (y)	60.2±1.3	59.7±2.6
Body weight (kg)	83.7±3.1	83.6±3.4
Height (m)	1.75±0.01	1.77±0.02
BMI (kg/m^2)	27.2±1.00	26.8±0.82
Fasting plasma glucose (mmol/L)	5.8±0.1	10.3±0.7*
Plasma glucose$OGTT_{120}$[2] (mmol/L)	6.1±0.4	19.7±0.8*#
Basal plasma insulin (pmol/L)	102.8±9.4	102.1±19.6
HbA1c (%)	5.6±0.1	8.1±0.3*
OGIS120[3] (mL/(min·m^2))	351±16	258±13*
Diagnosed with type 2 diabetes (y)	NA	9.2±1.44
Medication	NA	Metformin and/or SU-derivatives

Values are means±SEM, n=10 per group. [2] Plasma glucose concentrations after a 2h OGTT, [3] Oral glucose insulin sensitivity index. * Different from control group P<0.01. # Different from fasting values P<0.01.

Screening

All subjects performed an oral glucose tolerance test (OGTT). After an overnight fast, subjects arrived at the laboratory at 08.00 am by car or public transportation. A catheter (Baxter BV, Utrecht, the Netherlands) was inserted into an antecubital vein and a resting blood sample was drawn after which 75 g glucose (dissolved in 250 mL water) was ingested. After the bolus was consumed, blood was sampled every 30 min until 120 min. Plasma glucose concentrations were measured to determine glucose intolerance and/or type 2 diabetes according to the World Health Organization criteria of 1999[19]. In addition, plasma glucose and insulin concentrations were used to assess insulin

sensitivity using the oral glucose insulin sensitivity (OGIS)-index for a 2 hour OGTT as described by Mari et al.[20]

Design
Each subject participated in 3 trials, separated by at least 7 d, in which plasma glucose, insulin and amino acid responses were determined following the ingestion of 3 different beverages (CHO: carbohydrate, CHO+PRO: carbohydrate with a casein protein hydrolysate or CHO+PRO+LEU: carbohydrate, a casein protein hydrolysate and leucine). Subjects rested in a supine position in a reclining chair for 4 h. The test beverages were provided in a randomized order and double blind fashion.

Protocol
After an overnight fast, subjects reported to the laboratory at 08.00 am by car or public transportation. A Teflon catheter (Baxter BV, Utrecht, the Netherlands) was inserted into an antecubital vein for venous blood sampling and a resting blood sample was collected. At 0 min subjects drank a single bolus (4 mL/kg) of the experimental beverage. Blood samples were drawn every 15 min during the first hour after which blood was sampled at 30 min intervals until 240 min for measurement of plasma glucose and insulin concentrations. Plasma amino acid concentrations were determined at 1-hour intervals.

Diet and activity prior to testing
All subjects maintained their normal dietary and physical activity patterns throughout the entire experimental period. In addition, subjects refrained from heavy physical labor and/or exercise training for at least 3 d prior to each trial. Subjects filled out a food intake diary for 2 d prior to the first trial and were instructed to keep their dietary intake identical in the 2 days prior to the other 2 trials. Furthermore, the evening before each trial, all subjects received the same standardized meal (43.8 kJ/kg body weight; 60 Energy% (En%) carbohydrate, 28 En% fat and 12 En% protein).

Beverages
The subjects received a single bolus (4 mL/kg) containing 0.7 g/kg body weight (BW) carbohydrate (50% glucose and 50% maltodextrin, CHO) with 0.3 g/kg BW of a casein protein hydrolysate (CHO+PRO) or 0.3 g/kg BW of a casein protein hydrolysate and 0.1 g/kg BW of leucine (CHO+PRO+LEU). To investigate the modulating effect of additional protein hydrolysate ingestion, carbohydrate ingestion was kept identical in all trials. In accordance, free leucine was added in the CHO+PRO+LEU trial without reducing the amount of protein ingested, to evaluate whether the insulinotropic response to protein co-ingestion could be further enhanced. As such, the provided beverages were neither isocaloric nor isonitrogenous. Glucose and maltodextrin were obtained

from AVEBE (Veendam, the Netherlands), crystalline leucine from BUFA (Uitgeest, the Netherlands), and the casein protein hydrolysate was prepared by DSM Food Specialties (Delft, the Netherlands). The casein hydrolysate (Insuvital™) was obtained by enzymatic hydrolysis of sodium caseinate using a proprietary mix of proteases. Drinks were uniformly flavored by adding 0.2 g sodiumsaccharinate, 1.8 g citric acid, and 5 g cream vanilla flavor (Quest International, Naarden, the Netherlands) per liter beverage.

Blood sample analysis

Blood was collected in EDTA containing tubes and centrifuged at 1,000 g and 4°C for 10 min. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at -80°C until analyses. Glucose concentrations (Uni Kit III, Roche, Basel) were analyzed with the COBAS FARA semi-automatic analyzer (Roche). Plasma insulin was determined by radio immunoassay (HI-14K, Linco research Inc, St. Charles, USA). Free amino acids were analyzed using ion-exchange chromatography (JEOL, AminoTac JLC-500/V) with post-column ninhydrin derivatisation with norvaline as an internal standard. Prior to analysis samples were deproteinated with 5-sulphosalicylic acid. To determine glycosylated hemoglobin (HbA1c) content a 3 mL blood sample was collected in EDTA containing tubes and analyzed by high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany).

Statistics

Data are expressed as means±SEM. Primary outcome measures were plasma glucose, insulin and amino acid concentrations and plasma responses, calculated as areas under the curve above baseline values (AUC). To compare plasma metabolite concentrations over time and between trials, a two-way repeated measures analysis of variance (ANOVA) was applied. Changes over time within each group were tested using one-way repeated-measures ANOVA. Scheffé's post-hoc test was applied in case of a significant F-ratio to locate specific differences. Paired student's t-tests were used to compare fasting and 2 h OGTT values. Significance was set at the 0.05 level of confidence. All calculations were performed using StatView 5.0 (SAS Institute inc., Cary, NC, USA).

Results

Plasma insulin concentrations

Baseline plasma insulin concentrations were similar between groups and trials with mean values of 102.8±9.4 and 102.1±19.6 pmol/L for the type 2 diabetes and control group respectively (Figure 3.1A). In the type 2 diabetes patients, plasma insulin concentrations increased significantly in the CHO+PRO and the CHO+PRO+LEU trials only ($P<0.05$). In the control group, strong increases in

plasma insulin concentrations were reported in all trials (P<0.05). This increase was more pronounced in the CHO+PRO and the CHO+PRO+LEU trials, compared to the CHO trial. Insulin responses (expressed as AUC) in the diabetes group were 141±40 and 204±37% greater in the CHO+PRO and the CHO+PRO+LEU trials, respectively, compared to the CHO trial (P<0.05, Figure 3.1B). In the control group, insulin responses were 66±20 and 221±82% greater in the CHO+PRO and CHO+PRO+LEU trials respectively, compared to the CHO trial (P<0.05). Furthermore, in the control group the insulin response in the CHO+PRO+LEU trial was greater than the CHO+PRO trial (P<0.05). The insulin response did not differ between groups within the same trial.

Plasma glucose concentrations

Fasting plasma glucose concentrations were higher in the type 2 diabetes patients (8.6±0.6 mmol/L) compared to the normoglycemic controls (5.7±0.1 mmol/L, P<0.01). Following ingestion of the different beverages, plasma glucose concentrations remained significantly higher in the diabetes patients compared to their matched controls in all trials (P<0.01, Figure 3.2A). In the type 2 diabetes patients, plasma glucose concentrations significantly increased during the first h after beverage ingestion, after which concentrations returned to baseline levels (Figure 3.2A). In the control group, plasma glucose concentrations increased during the first 30 min after ingestion of the test drinks, after which concentrations returned to baseline values.

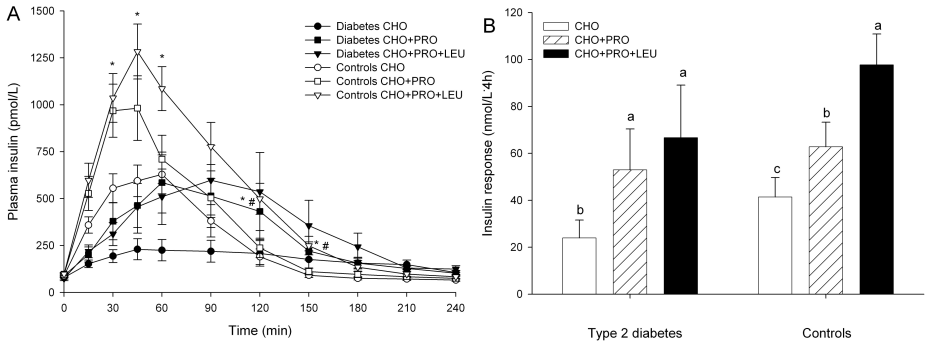

Figure 3.1. Plasma insulin concentrations (panel A) and responses (AUC, panel B) over a 4h period following the ingestion of carbohydrate (CHO), carbohydrate with a protein hydrolysate (CHO+PRO) and carbohydrate, protein hydrolysate and free leucine (CHO+PRO+LEU) in type 2 diabetes patients (T2D) and healthy control subjects (CON). Values are means ±SEM, n=10 per group. (A) *: significantly different compared to the CHO trial P<0.05, #: significantly different compared to the CHO+PRO trial, P<0.05. (B) Within a group, means without a common letter differ, P<0.05. No differences were found in responses between groups within the same trial.

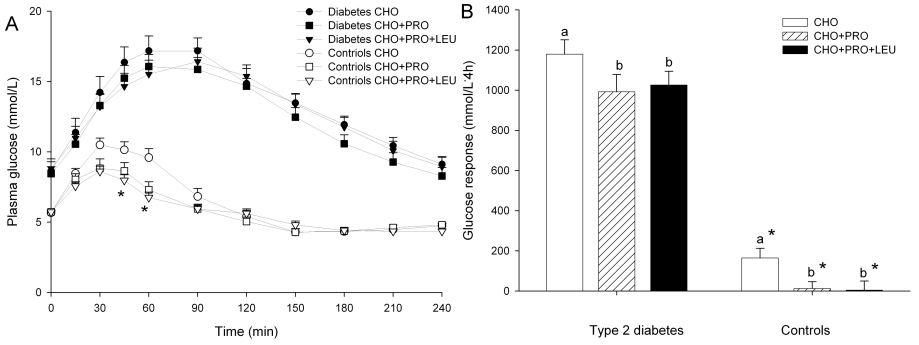

Figure 3.2. Plasma glucose concentrations (panel A) and response (AUC, panel B) over a 4h period following the ingestion of carbohydrate (CHO), carbohydrate with a protein hydrolysate (CHO+PRO) and carbohydrate, protein hydrolysate and free leucine (CHO+PRO+LEU) in type 2 diabetes patients (T2D) and healthy control subjects (CON). Values are means ±SEM, n=10 per group. (A) *: significantly different compared to the CHO trial P<0.05. (B) Within a group, means without a common letter differ, P<0.05. * Different from type 2 diabetes group within the same trial, P<0.05.

Furthermore, plasma glucose concentrations decreased faster in the CHO+PRO and the CHO+PRO+LEU trials compared to the CHO trial, resulting in lower plasma glucose concentrations at 45 and 60 min (P<0.05, Figure 3.2A). When expressed as AUC (Figure 3.2B), the glucose responses were reduced by 15±5 and 12±3% in the type 2 diabetes group and by 92±2 and 97±3% in the control group in the CHO+PRO and CHO+PRO+LEU trials, respectively, compared to the CHO trial (P<0.05). Plasma glucose responses were substantially higher in the diabetes patients when compared to the controls in all trials (P<0.01, Figure 3.2B). Glucose responses were inversely correlated with the accompanying insulin response in the type 2 diabetes patients (r=-0.48, P<0.01).

Table 3.2. Plasma amino acid concentrations in fasting ype 2 diabetes patients and normoglycemic control subjects

µmol/L	Controls	Type 2 diabetes
1-Methyl-histidine	9.5±1.3	9.4±1.4
3-Methyl-histidine	22.3±2.5	27.7±2.0
α-Aminobutyric acid	29.2±1.1	31.5±1.1
Alanine	370.3±19.2	431.1±17.0*
Arginine	128.1±7.0	110.2±3.5*
Asparagine	10.0±0.5	11.0±0.6
Aspartic acid	39.0±1.1	34.8±1.2*
Citrulline	48.9±1.6	43.7±3.1
Cystine	54.1±1.4	54.8±1.4
Glutamine	528.5±7.4	508.4±14.4
Glutamic acid	94.3±4.3	109.7±5.9*
Glycine	208.2±8.8	207.8±9.5
Histidine[2]	71.9±1.7	69.0±1.7
Isoleucine[2]	66.0±2.0	79.1±2.3*
Leucine[2]	122.8±3.0	144.9±3.2*
Lysine[2]	187.8±4.7	204.2±5.4*
Methionine[2]	21.7±0.6	20.6±0.7
Ornithine	50.4±1.6	51.7±1.4
Phenylalanine[2]	52.6±1.2	50.5±1.0
Proline	77.1±3.1	94.7±5.8*
Serine	92.7±1.7	90.1±3.0
Threonine[2]	112.0±3.7	118.6±4.6
Tryptophan[2]	41.3±2.3	37.5±1.9
Tyrosine	62.3±2.9	56.8±2.0
Valine[2]	216.7±5.0	252.4±4.9*

Values means±SEM, n=10 per group. [2] Essential amino acid. * Different from control group P<0.05.

Plasma amino acid concentrations

A complete overview of the subsequent plasma free amino acid responses, calculated as AUC, is provided in Table 3.3. Generally, amino acid responses were negative in the CHO trial, positive in the CHO+PRO trial and intermediate after leucine co-ingestion in the CHO+PRO+LEU trial. Strong correlations were observed between plasma insulin responses and the responses of plasma leucine ($r = 0.43$, $P<0.001$), citrulline ($r = 0.53$, $P<0.001$), cystine ($r = -0.27$, $P<0.04$), lysine ($r = 0.43$, $P<0.001$), methionine ($r = 0.27$,

P<0.04), ornithine (r = 0.33, P<0.01) and proline (r = 0.33, P<0.01) in both groups. In the type 2 diabetes group, the EAA-LEU response was negative in the CHO trial and significantly greater in the CHO+PRO and CHO+PRO+LEU trials (Figure 3.3A). Furthermore, the EAA-LEU response was lower (60±4%, P<0.05) in the CHO+PRO+LEU compared to the CHO+PRO trial. Plasma NEAA responses were negative in the CHO trial and were significantly greater in the CHO+PRO and CHO+PRO+LEU trials in the diabetes patients (P<0.05).
A negative plasma EAA-LEU response was observed in the CHO trial and significantly greater EAA- LEU responses were observed in the CHO+PRO and CHO+PRO+LEU trials compared to the CHO trial (Figure 3.3B).

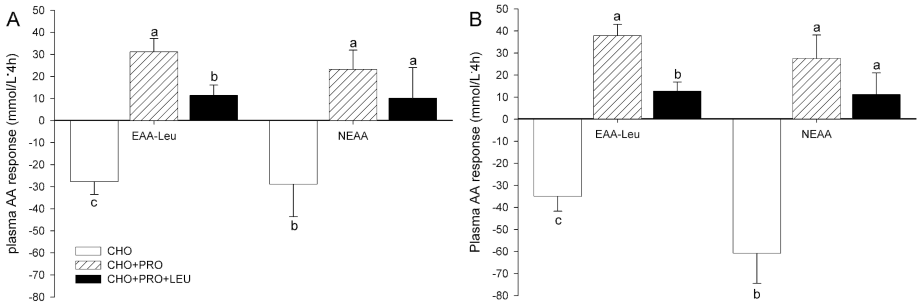

Figure 3.3. Plasma essential (without leucine, EAA-LEU) and non-essential amino acid (NEAA) responses, expressed AUC, over a 4h period following the ingestion of carbohydrate (CHO), carbohydrate with a protein hydrolysate (CHO+PRO) and carbohydrate, a protein hydrolysate and free leucine (CHO+PRO+LEU) in type 2 diabetes patients (A) and healthy control subjects (B). Values are means ±SEM, n=10 per group. Within a group, means without a common letter differ, P<0.05. No differences were found in responses between groups within the same trial.

Addition of leucine in the CHO+PRO+LEU trial resulted in a 65±5% lower plasma EAA- LEU response compared to the CHO+PRO trial (P<0.05). Plasma NEAA responses were negative in the CHO trial and were significantly greater in the CHO+PRO and CHO+PRO+LEU trials (P<0.05). Amino acid responses did not differ between the control and type 2 diabetes group.

Table 3.3. Plasma amino acid responses after beverage ingestion in type 2 diabetes patients and normoglycemic control subjects

AUC (mmol/L·4h)	Controls			Type 2 diabetes		
	CHO	CHO+PRO	CHO+PRO +LEU	CHO	CHO+PRO	CHO+PRO +LEU
1-Methyl-histidine	-0.2±0.1	-0.2±0.2	-0.1±0.1	-0.5±0.2	-0.2±0.1	-0.2±0.2
3-Methyl-histidine	-1.1±0.7	1.3±0.5 [a]	0.6±0.6	-1.8±0.6	-0.1±0.3*	-0.5±0.6
α-Aminobutyric acid	-0.6±0.3	0.1±0.3	-0.7±0.1	-0.5±0.2	-0.2±0.2	-0.7±0.3
Alanine	-0.9±5.7	13.0±4.9	5.8±4.5	5.4±5.2	11.8±4.8	3.7±6.3
Arginine	-3.1±1.3	0.4±1.0	-0.5±3.4	-2.7±1.8	0.3±0.9	0.7±1.3
Asparagine	-2.1±0.4	1.4±0.3[a]	0.2±0.2[a b]	-0.8±0.3*	1.2±0.4[a]	0.3±0.5
Aspartic acid	-0.2±0.2	0.3±0.1	-0.4±0.2	-0.3±0.3	0.4±0.2	-0.2±0.1
Citrulline	-3.8±0.5	-0.3±0.4[a]	1.1±0.2[a]	-3.4±0.8	-1.3±0.7	0.1±1.0
Cystine	-0.8±0.3	-0.8±0.4	-1.0±0.2	-0.3±0.3	-0.6±0.1	-0.7±0.3
Glutamine	-12.5±2.7	-2.7±1.4[a]	3.6±1.7[a]	-4.0±3.2	-1.5±2.6	-1.4±10.0*
Glutamic acid	-3.9±0.9	0.0±2.0	-4.7±1.4	-1.0±1.5	0.2±1.3	2.3±1.2
Glycine	-6.1±0.8	-3.8±0.9	-6.1±1.0	-4.0±1.7	-4.3±1.0	-7.1±1.8
Histidine[2]	-1.4±0.6	1.0±0.4[a]	-0.3±0.4	-1.7±0.5	0.2±0.6[a]	-1.0±0.6
Isoleucine[2]	-4.2±0.6	4.4±0.5[a]	-1.4±0.8[a, b]	-4.4±0.7	4.9±0.9[a]	0.7±0.8* [a ,b]
Leucine[2]	-7.5±1.1	5.6±0.9[a]	81.7±4.3[a ,b]	-7.9±1.0	5.4±1.3[a]	80.3±26.9[a,b]
Lysine[2]	-4.9±0.9	11.1±1.2[a]	11.4±1.1[a]	-4.2±1.2	8.2±1.9[a]	6.8±1.0[a]
Methionine[2]	-1.5±0.2	1.6±0.3[a]	0.9±0.3[a]	-1.0±0.2	1.5±0.2[a]	0.9±0.3[a]
Ornithine	-2.1±0.2	2.0±0.4[a]	2.8±0.3[a]	-2.5±0.4	1.7±0.3[a]	2.7±0.5[a]
Phenylalanine[2]	-2.3±0.4	1.7±0.4[a]	0.5±0.4	-1.5±0.3	1.1±0.2[a]	0.8±0.7[a]
Proline	-1.8±0.7	9.4±0.9[a]	6.7±0.9[a]	-2.4±0.8	9.5±1.1[a]	8.0±1.1[a]
Serine	-4.3±0.6	2.6±0.6[a]	-0.2±0.5[a ,b]	-1.8±0.5*	2.6±0.9[a]	0.0±1.4
Threonine[2]	-4.5±0.9	4.6±0.8[a]	1.3±0.7[a ,b]	-3.2±0.9	3.9±1.1[a]	1.2±1.5[a]
Tryptophan[2]	-2.3±0.8	1.7±0.9[a]	0.1±0.7	-2.1±0.9	0.5±0.3[a]	-1.6±0.7
Tyrosine	-3.3±0.5	5.0±0.7[a]	4.1±1.3[a]	-2.6±0.4	3.7±0.6[a]	3.3±0.7[a]
Valine[2]	-8.4±0.8	11.8±1.5[a]	0.2±1.2[a ,b]	-7.7±1.4	10.9±1.7[a]	3.5±1.3[a ,b]

Values means±SEM, n=10 per group. [2] Essential amino acid. * Different from control group within the same trials P<0.05. [a] Different from CHO trial within groups P<0.05. [b] Different from CHO+PRO trial within groups P<0.05.

Discussion

Numerous *in vitro* studies using primary pancreatic islet cells or β-cell lines have reported strong insulinotropic effects for arginine, leucine, isoleucine, alanine and phenylalanine but the various mechanisms by which amino acids can stimulate insulin secretion have not yet been fully elucidated[10]. Both *in vivo* and *in vitro* work has identified leucine as a particular interesting insulin

secretagogue, as leucine both induces and enhances pancreatic β-cell insulin secretion through its oxidative decarboxylation and its ability to allosterically activate glutamate dehydrogenase[7-10]. These findings seem to be in line with recent *in vivo* observations in healthy men, showing co-ingestion of relatively small amounts of free leucine to further augment the insulin response following the combined ingestion of carbohydrate and protein[17]. Furthermore, Xu et al.[9] suggested that the signals that stimulate insulin release are also responsible for the leucine-induced activation of the mammalian target of rapamycin (mTOR) signaling pathway in the pancreatic β-cell. The latter has been proposed to enhance β-cell function through the maintenance of β-cell mass. As such, leucine administration has been suggested as an excellent candidate to optimize the insulinotropic effects of protein co-ingestion. Therefore, we determined the postprandial plasma insulin, glucose and amino acid responses following co-ingestion of a casein protein hydrolysate with and without additional leucine together with a single, meal-like, bolus of carbohydrate in longstanding type 2 diabetes patients and healthy controls.

Ingestion of carbohydrate only (CHO) resulted in a blunted insulin response in the type 2 diabetes patients when compared to the normoglycemic controls, thereby clearly demonstrating the reduced sensitivity of the pancreas to glucose ingestion in the type 2 diabetic state[14]. Co-ingestion of the casein hydrolysate (CHO+PRO) resulted in a ~140 and ~70% greater insulin responses compared to the CHO trial in the type 2 diabetes patients and the normoglycemic controls, respectively. The additional administration of free leucine (CHO+PRO+LEU) further stimulated insulin release, resulting in a more than 200% greater insulin response in both the diabetes and control group, when compared to the CHO trial. The insulin responses in the CHO+PRO and CHO+PRO+LEU trials in the type 2 diabetes patients were of similar magnitude to those reported in the CHO trial and CHO+PRO trials in the healthy controls. Thus, even though the sensitivity of the pancreas to carbohydrate was significantly impaired in the longstanding type 2 diabetes patients, their capacity to secrete insulin in response to both glucose and amino acids is still highly functional. These data imply that the impaired insulin response following carbohydrate ingestion in type 2 diabetes patients is attributed to a reduced sensitivity of the β-cell to glucose, and does not necessarily represent an overall defect in the capacity of the pancreas to produce and/or secrete insulin.

The greater insulin response following protein or protein/leucine co-ingestion resulted in a reduced glucose response. The differences in glucose responses between the CHO and CHO+PRO or CHO+PRO+LEU trials were of similar magnitude in both groups. However, expressed in a relative manner, the reductions in the glucose response were 15±5 and 12±3% in the type 2 diabetes group, and 92±2 and 97±3% in the control group, respectively, compared to the CHO trial. These data extend on previous findings[15, 16], and show that protein/leucine co-ingestion represents an effective strategy to reduce

postprandial blood glucose excursions following the ingestion of a single bolus of carbohydrate, resembling the amount of carbohydrate in a low-fat meal. Consequently, our data imply that such nutritional interventions can be applied to improve postprandial blood glucose homeostasis in type 2 diabetes patients. It could be speculated that co-ingestion of protein/leucine with every main meal can improve blood glucose homeostasis over more prolonged periods. However, as daily food intake generally includes 3 main meals with various between-meal snacks, more studies are warranted to establish the potential of protein/amino acid co-ingestion as a strategy to improve blood glucose homeostasis under daily, free-living conditions.

Increasing postprandial insulin secretion could also have other benefits for the type 2 diabetes patient. Skeletal muscle protein breakdown rates have been reported to be elevated in uncontrolled type 2 diabetes[21]. The administration of protein and/or leucine with carbohydrate may also represent an effective strategy to prevent this loss of skeletal muscle mass by increasing postprandial insulin concentrations and by providing ample precursors for protein synthesis[22]. In addition, leucine administration has been suggested to stimulate muscle protein synthesis, by insulin-independent activation of the mTOR signaling pathway[23, 24]. Though we did not aim to assess the muscle protein anabolic response to protein and/or leucine co-ingestion, some interesting results were observed when evaluating the plasma amino acid concentrations. Whereas amino acid responses were negative after ingesting only carbohydrate, co-ingestion of the protein hydrolysate in the CHO+PRO trial resulted in a positive plasma amino acid response. Interestingly, additional supplementation with leucine (CHO+PRO+LEU) resulted in a ~60% lower plasma EAA-LEU response compared to the CHO+PRO trial, even though the same amount of protein was ingested. These findings seem to be in accordance with Nair et al.[25, 26] showing leucine administration to reduce most plasma amino acid concentrations. The lower amino acid responses in the CHO+PRO+LEU trial could be attributed to the proposed capacity of leucine to inhibit proteolysis and/or to stimulate protein synthesis[17, 23]. In addition, the lower amino acid response could also be explained by the effects of elevated insulin levels on amino acid oxidation and/or on splanchnic sequestration of amino acids. Clearly, more research is warranted to determine the effects of leucine administration on muscle protein balance *in vivo* in humans.

We conclude that co-ingestion of a protein hydrolysate with or without additional leucine augments endogenous insulin secretion following the consumption of a single bolus of carbohydrate, thereby substantially reducing postprandial blood glucose excursions in longstanding type 2 diabetes patients. Co-ingestion of a protein hydrolysate and/or leucine mixture represents an effective strategy to improve postprandial blood glucose homeostasis in type 2 diabetes patients.

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Chapter 4

Glycemic instability is an underestimated problem in type 2 diabetes

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Clin Sci (Lond), 2006; 111(2):119-26

Abstract

Objective: To assess the level of glycemic control by the measurement of 24 h blood glucose profiles and standard blood analyses under identical nutritional and physical activity conditions in type 2 diabetes patients and healthy, normoglycemic controls.
Design: A total of 11 male, type 2 diabetes patients and 11 healthy, matched controls participated in a 24 h continuous subcutaneous glucose monitoring (CGMS) assessment trial under strictly standardized dietary and physical activity conditions. In addition, fasting plasma glucose, insulin and HbA1c concentrations were measured, and an oral glucose tolerance test was performed to calculate indices of whole-body insulin sensitivity, oral glucose tolerance and/or glycemic control.
Results: In the healthy control group, hyperglycemia (blood glucose concentration >10 mmol/L) was hardLy present (2±1% or 0.4±0.2 / 24 h). However, in the type 2 diabetes patients hyperglycemia was experienced for as much as 55±7% of the time (13±2 h / 24 h) while using the same standardized diet. Breakfast-related hyperglycemia contributed most (46±7%, ANOVA, $P<0.01$) to the total amount of hyperglycemia and postprandial glycemic instability. In the diabetes patients, blood HbA1c contents correlated well with the duration of hyperglycemia and the postprandial glucose responses ($P<0.05$).
Conclusions: CGMS measurements show that standard measures for glycemic control underestimate the amount of hyperglycemia prevalent during real-life conditions in type 2 diabetes. Given the macro- and microvascular damage caused by postprandial hyperglycemia, CGMS provides an excellent tool to evaluate alternative therapeutic strategies to reduce hyperglycemic blood glucose excursions.

Introduction

Over the last 15 years, improvements in microdialysis and biosensor technology have enabled clinicians to reliably monitor plasma and/or interstitial glucose concentrations in an ambulatory and continuous way[1, 2]. These, so-called, continuous subcutaneous glucose-monitoring systems (CGMS) have proven quite useful to optimize individual exogenous insulin administration in diabetes patients[3], since they provide information on ambulatory postprandial[4] and/or nocturnal glucose excursions[5]. Moreover, both in children and adults with type 1 diabetes it has been shown that average 24 h blood glucose concentrations strongly correlate with HbA1c concentrations[6, 7]. However, the inter- and intra-individual day-to-day variation in glycemic load, meal composition[8] and daily physical activity[9] can complicate therapeutic decision-making based on these 24 h blood glucose profiles[10, 11]. Therefore, in order to compare CGMS results between normoglycemic and diabetic subjects, standardization of both diet[8] and physical activity[9] is essential.

Epidemiological studies and preliminary intervention studies have shown that postprandial hyperglycemia is a direct and independent risk factor for the development of cardiovascular disease (CVD)[12]. Importantly, the postprandial rapid increase in blood glucose concentrations or 'hyperglycemic spikes' seem to be even more relevant to the onset of cardiovascular complications than merely elevated fasting plasma glucose[13]. Therefore, therapeutic targets should be aimed at reducing postprandial blood glucose excursions. Although scientific studies on the prevalence of hyperglycemic spikes in type 2 diabetes are still scarce[13], recommendations on proper glycemic control have recently been redefined[14, 15]

To define abnormal postprandial blood glucose excursions and relate this to the pathogenesis of diabetic vascular complications, it is important to have more detailed information on normal postprandial blood glucose profiles in a non-insulin resistant population under exactly the same dietary ambulatory conditions. Therefore, in the present study, we investigated 24 h blood glucose profiles in type 2 diabetes patients on oral blood glucose lowering medication and healthy, normoglycemic controls under strictly standardized, but free-living conditions. As such, this study provides a frame of reference for future studies on the role of real-life postprandial hyperglycemia in the pathogenesis of diabetic complications.

Subjects and methods

Subjects

A total of 11 longstanding male type 2 diabetes patients and 11 healthy, age and BMI matched, normoglycemic control subjects were selected to participate in this study. Subjects' characteristics are presented in Table 4.1. Exclusion criteria were impaired renal or liver function, severe obesity (BMI>35 kg/m^2), cardiac

disease, hypertension, diabetic complications, and exogenous insulin therapy. All type 2 diabetes patients were treated with oral plasma glucose lowering medication (metformin only (n=3), or in combination with sulfonylureas (n=8)). All medication was continued during the trials. All subjects were informed about the nature and the risks of the experimental procedures before their written informed consent was obtained. The study was approved by the local Medical Ethical Committee.

Table 4.1. Subject characteristics

	Controls	Type 2 diabetes
Age (yrs)	59±2	58±1
BMI (kg/m^2)	27.8±1.4	27.9±1.2
Years type 2 diabetes	NA	8±1
HbA1c (%)	5.5±0.1	7.4±0.3*
Fasting glucose (mmol/L)	5.7±0.2	10.6±1.0*
HOMA-IR	3.45±0.5	8.0±1.4†
$OGIS_{120}$ (ml/min/m^2)	367±19	256±19*

All values are expressed as means±SEM. *: significantly different between groups; P<0.01, †: P<0.001.

Screening

Before inclusion, all subjects first performed an oral glucose tolerance test (OGTT). Blood glucose lowering medication was withheld prior to the screening. After an overnight fast, subjects reported at the laboratory at 8.00 a.m. A catheter (Baxter BV, Utrecht, the Netherlands) was inserted into an antecubital vein and a resting blood sample was drawn after which a bolus of 75 g glucose (dissolved in 250 mL water) was ingested (t= 0 min). After the bolus was consumed, blood was sampled every 30 min until t=120 min. Plasma glucose concentrations were measured to determine glucose intolerance and/or type 2 diabetes according to the World Health Organization criteria of 1999[16]. In addition, plasma glucose and insulin concentrations were used to assess insulin sensitivity (IS) using the oral glucose insulin sensitivity (OGIS)-index for a 2 h OGTT as described by Mari et al.[17] and whole-body insulin resistance using the homeostasis model assessment insulin resistance index (HOMA-IR)[18].

Blood sample analysis

Blood (10 mL) was collected in EDTA containing tubes and centrifuged at 1,000 g and 4°C for 10 min. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at -80°C until analyses. Glucose concentrations (Uni Kit III, Roche, Basel) were analyzed with the COBAS FARA semi-automatic analyzer (Roche). Plasma insulin was determined in duplicate and averaged by radioimmunoassay (HI-14K, Linco research Inc, St. Charles, USA). To determine HbA1c content a 3 mL blood sample was collected in EDTA

containing tubes and analyzed by high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany).

Study protocol

All experimental trials described in this study are part of a greater project investigating the effects of nutritional interventions to improve glycemic control in type 2 diabetes patients. On the first day subjects reported to the laboratory in the afternoon and were instructed about their diet, and on the use of the food intake and physical activity diaries. Next, subjects received instructions in the use of the capillary blood sampling method (Glucocard Memory PC, A. Menarini Diagnostics, Firenze, Italy) used for the calibration of the continuous glucose monitoring system. All subjects were instructed to measure capillary blood glucose concentrations before every meal. After the subjects were fully instructed, a microdialysis fiber (Medica, Medolla, Italy) with an internal diameter of 0.17 mm and a cut-off weight of 18 kD was inserted in the peri-umbilical region, without anesthesia, using an 18-gauge Teflon catheter as a guide, as described previously[19]. For the measurements the micro-fiber was then connected to a portable CGMS (GlucoDay®S, A. Menarini Diagnostics, Firenze, Italy), which consists of a peristaltic pump that pumps Dulbecco's solution at 10 µL/min through the microdialysis fiber. A more detailed description of the device has been published earlier[1]. Briefly, the subcutaneous interstitial fluid is taken up by the microdialysis fiber and is transported to the measuring cell. The glucose sensor, consisting of immobilized glucose oxidase, measures the glucose concentration every second and stores an average value every 3 min for a total of 48 h. The entire device weighs about 250 g and is worn in a pouch under the subjects' clothes. After the CGMS was checked for proper function, subjects were provided with their diet (pre-weighed and packaged meals, drinks and snacks) and were allowed to return home and resume all their normal activities. CGMS data of the second test day (from 7.00 am to 7.00 am) were used for data analysis. The first period was used to familiarize subjects with the equipment and, therefore, not used in the data analyses.

Diet and physical activity

All subjects maintained their normal physical activity patterns throughout the entire experimental period. Subjects refrained from heavy physical labor and exercise training for at least 3 d prior to and on the day of the trial. Subjects were asked to keep a comprehensive record of time spent performing al activities (to the nearest 10 min) including sleeping, eating, sitting, standing, watching television, occupational activity and household tasks, as well as information on the duration and relative intensity (e.g. light, moderate) of all structured activities. The rate of energy expenditure for each activity was then determined using the Compendium of Physical Activities[20]. Daily energy expenditure did not differ between groups and averaged 13.6±0.7 and 13.3±0.6

MJ day^{-1} in the type 2 diabetes patients and normoglycemic controls, respectively. All meals, snacks and beverages were provided in pre-weight packages and ingested at pre-determined time points to ensure fully standardized dietary modulation. On the evening prior to the 24h analyses period, all subjects received the same standardized meal (43.8 kJ kg^{-1} BW; consisting of 60 Energy% (En%) carbohydrate, 28 En% fat and 12 En% protein). The following day the subjects were instructed to ingest their designated meals, drinks and snacks at set time-points. Throughout this 24 h test period subjects received a standardized diet (3 meals and 3 snacks per day) representing an energy intake of 121 kJ kg^{-1} BW per day consisting of 64 En% carbohydrate, 25 En% fat and 11 En% protein. Before and after consuming a meal (i.e. breakfast, lunch and dinner) subjects were asked to obtain a capillary blood glucose sample (Glucocard Memory PC). The following day the subjects reported back to our laboratory to obtain a non-fasting venous blood glucose measurement and to remove the CGMS. The acquired data were then downloaded from the device to a personal computer with GlucoDay® software (V3.0.5). Values reported by the CGMS were converted into glucose values using the capillary glucose measurements as calibration values.

Statistics and data analyses

Data are expressed as means±SEM. Glucose responses were calculated as mean glucose area under the curve (AUC) up to 6 h after each meal. Since the CGMS device provides an average glucose value every 3 min, AUC is expressed as mmol/L * 3 min. To quantify and compare the glucose excursions in the control and diabetes population, AUC and the amount of time during which glucose concentrations were above 10.0 mmol/L or below 3.9 mmol/L were calculated. On the first and second study day, fasting glucose was determined from the calibrated CGMS curves 10 min before breakfast and averaged. The non-fasting venous blood glucose measurement was used to calculate the coefficient of variation (CV) of the CGMS data. Relationships between CGMS parameters and standard measures of IS were calculated using linear regression models.

To assess intra-day glycemic variability, continuous overall net glycemic action (CONGA), a novel method recently described by McDonnell et al[21] was used. CONGAn has been defined as the standard deviation of the differences in glucose concentration using varying time differences of n hours. We used CONGA1, CONGA2 and CONGA4, indicating intra-day glycemic variability based on 1 h, 2 h and 4 h time differences, respectively. In normal non-diabetic subjects CONGA values vary between 0.4 and 1.2, while values above 1.5 indicate glycemic lability[21].

Before pooling data from all 22 subjects, homogeneity of regression was tested using ANCOVA in order to exclude significant interaction. Time dependent variables were tested using repeated-measures ANOVA with a Tukey-Kramer post-hoc test when applicable. For non-time dependent variables, a Student's t-

test for unpaired observations was applied. Significance was set at the 0.05 level of confidence. All statistical calculations were performed using the SPSS 12.0.1 software package (SPSS Inc, Chicago, IL, USA).

Results

Baseline and postprandial blood glucose responses are provided in Table 4.2. Total 24 h blood glucose concentrations in both diabetes patients and healthy controls are illustrated in Figure 4.1. Basal and mean glucose concentrations were significantly greater in the type 2 diabetes patients vs the normoglycemic controls (t-test, $p<0.05$). In the type 2 diabetes patients, the prevalence of hyperglycemia (>10.0 mmol/L) was 55±7% of the 24 h period. In contrast, in the normoglycemic controls, hyperglycemia was evident in 1.6±1%. As such, hyperglycemia was present for 13.3±1.7 h and 0.38±0.2 h, respectively.

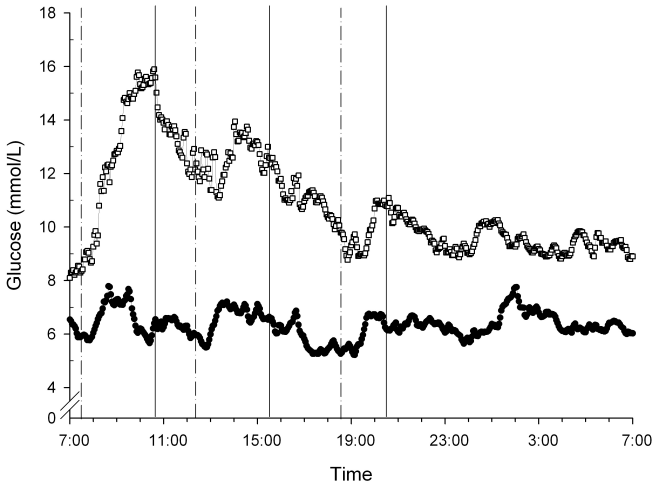

Figure 4.1. Mean±SEM glucose concentrations from 07:00 till 07:00 h using CGMS in respectively, eleven healthy, control subjects (lower curve) and eleven type 2 diabetes patients (upper curve). The SEM is indicated by the gray bars. The vertical lines indicate the time that subjects were consuming their standardized dietary components, consisting of breakfast (BrFst) (07:00-07:30), morning snack (10:30-11:00), lunch (12:30-13:00), afternoon snack (15:30-16:00), dinner (18:30-19:00) and evening snack (20:30-21:00), respectively.

The postprandial AUCs above 10.0 mmol/L following breakfast, lunch and dinner contribute, respectively 46±7%, 29±3%, 11±3% to the total amount of hyperglycemia in our type 2 diabetes patients present during the 24 h monitoring period. This breakfast-related hyperglycemia was significantly greater (ANOVA, $p<0.01$) compared to the amount of hyperglycemia during the evening or during the night.

Both in type 2 diabetes patients and healthy controls the average CONGA1 values following breakfast were significantly raised compared to the 6 h following lunch and dinner (Table 4.2, ANOVA, $p<0.01$). CONGA1 values were

lowest during the night (ANOVA, p<0.01) and did not differ between groups from 01.00 – 06.00 h (ANOVA, p>0.05, Table 4.2).
In this study the coefficient of variation (CV) between interstitial CGMS glucose values and venous blood glucose was on average 8.0±1.3%.

Table 4.2 CGMS measurements

	Control subjects (n=11)	Diabetes patients (n=11)
24 h analysis (7-7 am)		
Mean 24h glucose (mmol/L)	6.3 ± 0.2	10.8 ± 0.5*
Hyperglycemic episodes (h)	0.4 ± 0.2	13.3 ± 1.7*
Hypoglycemic episodes (h)	0.5 ± 0.2	0.1 ± 0.05**
FPG (mmol/L)	5.9 ± 0.4	8.6 ± 0.6*
Mean nocturnal glucose (mmol/L)	7.0 ± 0.9	9.3 ± 0.8*
CONGA1	1.5 ± 0.1	2.5 ± 0.1*
CONGA2	1.7 ± 0.1	3.4 ± 0.1*
CONGA4	1.8 ± 0.2	4.2 ± 0.2*
Postprandial analyses		
AUC PP breakfast	778 ± 38	1559 ± 75*
AUC PP lunch	713 ± 20	1419 ± 80*
AUC PP dinner	736 ± 39	1158 ± 82*
Glycemic variability		
CONGA1 PP breakfast	1.8 ± 0.3	3.4 ± 0.3#
CONGA1 PP lunch	1.5 ± 0.2	2.2 ± 0.2$
CONGA1 PP dinner	1.5 ± 0.2	2.0 ± 0.3$
CONGA1 nocturnal fasting	1.0 ± 0.2	1.2 ± 0.3

Data presented are means±SEM.; * significant group difference, P<0.001, # = P<0.01, $ = P<0.05, ANOVA; Hyperglycemic episodes, total time during which [glucose] levels are above 10.0 mmol/L; Hypoglycemic episodes, total time during which [glucose] levels are below 3.9 mmol/L; Mean Noct glucose, average glucose concentration between 24:00 and 07:00 h; CONGA1,2,4: continuous overall net glycemic action describing intra-day glycemic variability between respectively 1, 2 and 4 h time periods over 24 h ; AUC PP, area under the curve 6 h postprandial (mmol/L/6 h); CONGA1 glycemic variability between 1 h time periods.

Correlations between CGMS parameters and our standard measures for glycemic control are presented in Table 4.3. In the diabetes patients, HbA1c-levels correlated well with the average 24 h blood glucose concentrations (r=0.81, P<0.01), the time during which blood glucose levels were >10 mmol/L (r= 0.70, P<0.05), and postprandial AUC following lunch (r=0.80, P<0.01) and dinner (r=0.87, P<0.01). In a subgroup of diabetes patients with apparent acceptable glycemic control (HbA1c ≤ 7.0, n=6), hyperglycemia was present for 46±8% of the day (11.0±1.9 h).

Both in the diabetes and control group, mean 24 h and nocturnal blood glucose concentrations correlated strongly with fasting plasma glucose levels (R between 0.61-0.86, P<0.05). In both groups, no significant correlations were reported between the 24 h CONGA indices and HbA1c content, however, in the diabetes patients a significant correlation was found between postprandial CONGA1 values and AUC in the 6 h following a meal (r=0.47, p<0.01). When pooling the data from both groups, the 24 h CONGAn values correlated significantly with blood HbA1c content (r=0.53-0.66, P<0.01), mean 24 h glucose concentrations (r=0.73-0.77, P<0.001) and to a lesser extent with mean fasting plasma glucose concentrations (r=0.50-0.52, P<0.05). Also, a significant correlation was found between postprandial CONGA1 values and AUC 6 h following a meal (r=0.60, p<0.001)

Table 4.3 Pearson's correlation matrix between standard insulin sensitivity measures and CGMS measures in both diabetes patients and control subjects

Variable	mean 24 h glucose	% hyper-glycemia	mean nocturnal glucose	AUC PP breakfast	AUC PP lunch	AUC PP dinner
Diabetes patients (n=11)						
FPG (mmol/L)	0.61*	0.50	0.70*	0.01*	0.35	0.41
HbA1c (%)	0.81†	0.70*	0.30	0.37	0.80†	0.87†
Control Subjects (n=11)						
FPG (mmol/L)	0.84†	0.37	0.86†	0.45*	0.40	0.71*
HbA1c (%)	0.05	-0.18	0.03	-0.17	0.32	0.01*

Data presented are means±SEM. *significant correlations P<0.05, † P<0.01. %Hyperglycemia., percentage of time glucose concentration above 10.0 mmol/L; FPG, fasting glucose was determined from the calibrated CGMS curves 10 min before breakfast on the second day; HbA1c, glycated hemoglobin;%Hyperglycemia, percentage of time [glucose] above 10.0 mmol/L; mean noct glucose, average glucose concentration between 00:00 am and 07:00 h; AUC PP, area under the curve 6h postprandial (mmol/L/6 h).

Discussion

The present study shows that under normal, standardized dietary conditions, type 2 diabetes patients using oral blood glucose lowering medication experience a substantial amount of hyperglycemia for more than 13 h within a 24 h period. This disturbance in blood glucose homeostasis is predominantly present following breakfast. After comparing 24 h blood glucose profiles between healthy, normoglycemic controls and type 2 diabetes patients under usual medical care by a general practitioner, it seems clear that standard treatment schemes with oral blood glucose lowering drugs appear to have insufficient therapeutic strength to normalize postprandial hyperglycemia. Given the clinical relevance of the hyperglycemic spikes[13], CGMS provides an excellent tool to evaluate the level of glycemic stability in type 2 diabetes patients.

The concept that oral blood glucose lowering therapy provides inadequate protection against hyperglycemia is not new[22-24]. Epidemiological studies and preliminary intervention studies have shown that postprandial hyperglycemia is a direct and independent risk factor for the development of cardiovascular disease[12]. However, the postprandial rapid increase in blood glucose concentrations seems to be more relevant to the onset of cardiovascular complications than merely elevated fasting plasma glucose concentrations[13]. Therefore, more detailed information on 24 h blood glucose profiles in a diabetic state is essential to increase our understanding of the relationship between hyperglycemia, glucotoxicity and cardiovascular morbidity. In an attempt to assess postprandial glycemic instability in type 2 diabetes, we applied CGMS in diabetes patients and compared this with blood glucose profiles of normoglycemic subjects under strict nutritional and exercise standardization, but otherwise free-living conditions. In most of our normoglycemic subjects, hyperglycemia or glycemic instability was not detectable. In contrast, despite healthy dietary conditions and continued use of oral blood glucose lowering medication according to standard primary care[25] and international guidelines[15], the type 2 diabetes patients were hyperglycemic during more than 13 h per day, while using exactly the same diet as the normoglycemic controls. In accordance with earlier observations by Monnier et al.[26], this study shows that postprandial hyperglycemia was most prominent following breakfast and less evident during the night. As we provided a healthy, balanced diet (43.8 kJ/kg BW; consisting of 60 En%), carbohydrate, 28 En% fat and 12 En% protein), it could be speculated that the total amount of hyperglycemia may even be worse under normal, unrestricted dietary conditions. The observed levels of hyperglycemia during the day (13±2 h / 24 h) are unacceptable and likely cause the excess formation of advanced glycation end-products[27], causing the macro- and microvascular damage[28].

In line with earlier studies[29-31], our findings emphasize the need for different types of interventional strategies in type 2 diabetes patients. It should be noted

that there is a weak, non-significant, correlation between fasting blood glucose and the percentage of hyperglycemia (r=0.25, p>0.05, Table 4.3). The latter indicates that FPG is unlikely to be of sufficient sensitivity to successfully evaluate new treatment strategies that focus on reducing postprandial hyperglycemia. For more long-term evaluation purposes, changes in blood HbA1c concentrations have generally been assessed, since blood HbA1c content correlates relatively well with both mean 24 h[32, 33] and postprandial glucose levels[6, 7]. In accordance, in the present study we observed strong correlations between HbA1c and mean 24 h glucose and postprandial glucose levels following lunch and dinner (Table 4.3). It should be mentioned here that even under clinically acceptable HbA1c levels (i.e. HbA1c ≤ 7.0 in 6 out of 11 diabetes patients) hyperglycemia can still be unacceptably large at 11±2 hours of blood glucose excursion >10 mmol/L per 24 h. Therefore, these results extend on earlier findings[24, 34, 35], and strongly suggest that the ability of HbA1c to monitor postprandial hyperglycemia is debatable. Moreover, the measurement of prospective changes in blood HbA1c content only has sufficient sensitivity to detect changes in glucose homeostasis during middle to long-term interventions[36]. Therefore, the present study underlines the notion that CGMS is a promising tool when evaluating short-term (<3 months) changes in blood glucose homeostasis following pharmacological, dietary and/or exercise interventions[1].

Another benefit of the CGMS approach, that has potential clinical application as well, is the possibility to calculate the level of glycemic instability in insulin resistant states. This so-called Continuous Overall Net Glycemic Action (CONGAn) is probably a more appropriate measure to assess short-term changes in glucose homeostasis throughout the day[21]. This CGMS measure reflects the standard deviation of the differences in glucose concentration using varying time windows[21]. Therefore, we determined CONGAn values in both our diabetes patients and normoglycemic controls (Table 4.2). The proposed sensitivity of CGMS to detect subtle variations in glycemic control was confirmed in our normoglycemic control group. Interestingly, 2 of our control subjects appeared to have rather high postprandial CONGA1 values that almost approached values observed in the type 2 diabetes patients (i.e. average postprandial CONGA1 >2.1). These 2 subjects also showed the highest insulin values during the oral glucose tolerance test, and were the only 'normoglycemic' persons who showed some hyperglycemia throughout the day (data not shown). Altogether, our results suggest that more advanced CGMS analyses techniques provide promising measures to assess glycemic instability in diabetes patients[21]. Research is warranted to investigate the diagnostic value of CGMS in other diabetes related populations, like patients in a pre-diabetic and/or insulin resistant state.

In conclusion, detailed analyses of 24 h blood glucose profiles show that standard measures for glycemic stability grossly underestimate the amount of

hyperglycemia during real-life conditions in type 2 diabetes patients. Given the macro- and microvascular damage caused by postprandial hyperglycemia, CGMS provides an excellent tool to more directly evaluate additional therapeutic strategies to reduce the amount of glycemic instability and risk of cardiovascular complications in type 2 diabetes patients.

Acknowledgments
The authors would like to thank A. Menarini Diagnostics BENELUX and Hanneke van Milligen for the technical support. We gratefully acknowledge our volunteers for participating in the experimental trials.

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Chapter 5

Protein hydrolysate/leucine co-ingestion reduces the prevalence of hyperglycemia in type 2 diabetes patients

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Diabetes Care, 2006; 29(12):2721-2

Introduction

Epidemiological surveys and preliminary intervention studies have shown that postprandial hyperglycemia is a direct and independent risk factor for the development of cardiovascular disease in type 2 diabetes[1-4]. Moreover, it has been reported that postprandial spikes in the blood glucose concentration are even more relevant to the onset of cardiovascular complications than merely elevated fasting blood glucose levels[5-7].

Protein hydrolysate/leucine co-ingestion could represent an effective nutritional intervention to stimulate postprandial insulin secretion, augment postprandial blood glucose disposal and improve blood glucose homeostasis in type 2 diabetes patients[8-10]. Though promising, the clinical relevance and applicability of such a nutritional intervention remains to be established.

In the present study, we applied continuous glucose monitoring to asses the impact of protein hydrolysate/leucine co-ingestion with each main meal as a nutritional intervention strategy to improve daily glycemic control in longstanding type 2 diabetes patients.

Research and methods

Eleven longstanding type 2 diabetes patients (age:58±1y; BMI:28±1kg/m^2; HbA1c:7.4±0.3%) and eleven matched, healthy controls (age:59±2y; BMI:28±1kg/m^2; HbA1c:5.5±0.1%) participated in this study. All subjects were screened for type 2 diabetes according to ADA guidelines[11]. Type 2 diabetes patients had been diagnosed for 8±1 y and were using metformin with (n=8) or without (n=3) a sulfonylurea derivative. Blood glucose lowering medication was continued throughout the experimental trials. Subjects maintained normal dietary and physical activity patterns, but refrained from exhaustive physical labor and exercise training for 3d prior to each trial.

Each subject participated in a cross-over study with 2 trials, during which blood glucose concentrations were recorded for 40h under free-living conditions using a continuous glucose monitoring system (GlucoDay®S, A. Menarini Diagnostics, Firenze, Italy)[12]. Subjects received 3 beverages containing a protein hydrolysate/leucine mixture or a placebo beverage. Pre-packed beverages were consumed directly after each main meal. All meals, snacks and beverages were provided in pre-weighed packages and were ingested at pre-determined time-points to ensure fully standardized dietary modulation during both trials. The standardized diet provided 121 kJ/kg per day (64 En% carbohydrate, 25 En% fat and 11 En% protein). Beverages contained 4 mL/kg water (PLA) or water containing 0.3 g/kg casein protein hydrolysate and 0.1 g/kg leucine (PRO). Both trials were performed in a randomized and double blind manner.

The acquired data were downloaded from the continuous glucose monitoring system (CGMS) onto a personal computer with GlucoDay® software (V3.0.5). Values reported by the CGMS were converted into glucose values using SMBG

values. To quantify and compare the prevalence of hyperglycemia between groups and trials, the amount of time during which glucose concentrations were >10 mmol/L was calculated. A multi-way ANOVA or a Student's t-test for paired or unpaired observations were applied where applicable. All data are expressed as means±SEM and significance was set at P<0.05.

Results

Total 24h glucose concentrations were higher in the diabetes vs the control group (10.2±0.4 vs 6.2±0.6 mmol/L, respectively; P<0.01; Figure 5.1). In the diabetes patients, 24h glucose concentrations in the PRO trial were significantly lower compared to the PLA trial (9.6±0.6 vs 10.8±0.5 mmol/L, respectively; P<0.05). PRO ingestion resulted in a 11±3% decline in the overall glucose response in the diabetes patients (P<0.05). In the control group, differences between trials did not reach statistical significance (6.2±0.4 vs 6.3±0.2 mmol/L).

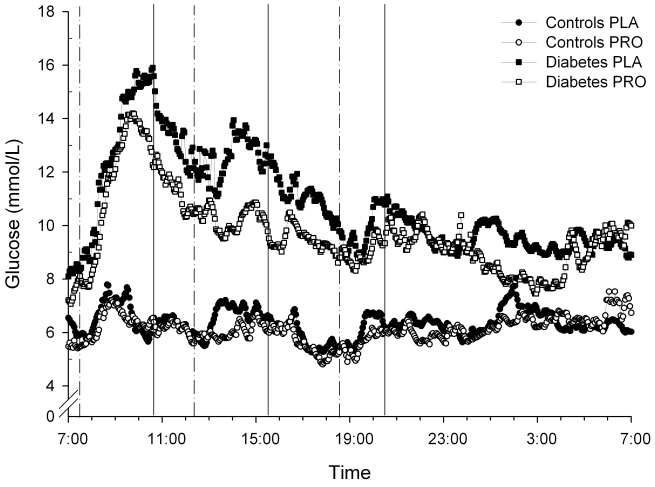

Figure 5.1. Mean blood glucose patterns over a 24h period while using a standardized diet with (open symbols) or without (filled symbols) co-ingestion of a protein hydrolysate/leucine mixture with each main meal in longstanding type 2 diabetes patients (■/□, n=11) and healthy, matched controls (●/○, n=11). Vertical dashed lines indicate time of breakfast (07:30), lunch (12:30) and dinner (18:30), respectively. Solid lines indicate between-meal

Despite a healthy, standardized diet and the continued use of oral blood glucose lowering medication, hyperglycemia (>10 mmol/L) was prevalent during 55±7% of the 24h period in the diabetes patients in the PLA trial. In the control group, hyperglycemia was hardLy present (2±1% or 25'±15' min). In the diabetes patients, the prevalence of hyperglycemia was significantly lower in the PRO vs PLA trial (39±6% vs 55±7%, respectively; P<0.05). The latter represented a 26±9% reduction in the prevalence of hyperglycemia from 13:18'±1:40' h in the PLA trial, to 9:42'±1:54' h in the PRO trial (P<0.05).

Conclusions

The prevalence of elevated postprandial glucose excursions in type 2 diabetes patients imposes a direct and independent risk for the development of cardiovascular complications[6, 7, 13]. In accordance, both the Diabetes Control and Complications Trial[1] and the U.K. Prospective Diabetes Study[2-4] report that improving glycemic control effectively reduces the risk of developing micro- and macrovascular complications and cardiovascular disease.

The present study shows that longstanding type 2 diabetes patients who receive standard primary medical care experience hyperglycemia throughout the greater part of the day. Co-ingestion of a protein hydrolysate/leucine mixture following each main meal substantially reduces the prevalence of hyperglycemia in these patients. This is accompanied by a significant reduction in average 24h blood glucose concentration. Although the use of continuous glucose monitoring devices in an applied setting does not allow for concomitant insulin measurements, numerous other studies[8-10, 14, 15] have repeatedLy shown that protein hydrolysate/amino acid co-ingestion stimulates insulin secretion resulting in an improved glucose disposal and reduced postprandial glucose concentrations.

These data extend on previous findings and provide evidence that protein hydrolysate/leucine co-ingestion represents an effective dietary strategy to improve daily blood glucose homeostasis under free-living conditions in longstanding type 2 diabetes patients.

Acknowledgments

The described work was supported by a grant from DSM Food Specialties (Delft, The Netherlands). We thank A. Menarini Diagnostics BENELUX and Hanneke van Milligen for her technical assistance. This study was presented at the 66th American Diabetes Association in Washington DC, June 2006.

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Chapter 6

Protein co-ingestion does not modulate 24 h glycemic control in longstanding type 2 diabetes patients

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Eur J Clin Nutr, 2007; Aug 22

Abstract

Objective: Evaluate the efficacy of protein hydrolysate co-ingestion as a dietary strategy to improve blood glucose homeostasis under free-living conditions in longstanding type 2 diabetes patients.
Design: A total of 13 type 2 diabetes patients were enrolled in a randomized, double blind cross-over design and studied on 2 occasions for 40 h under strict dietary standardization but otherwise normal, free-living conditions. In one trial, subjects ingested a protein hydrolysate (0.4 g/kg bw casein hydrolysate, PRO) with every main meal. In the other trial, a placebo was ingested (PLA). Blood glucose concentrations were assessed by continuous glucose monitoring.
Results: Average 24 h glucose concentrations were similar between the PLA and the PRO trials (8.9±0.8 mmol/L vs 9.2±0.7 mmol/L, respectively). Hyperglycemia (glucose concentrations >10 mmol/L) was experienced 34±9% of the time (8±2 h/24 h) in the PLA trial. protein hydrolysate co-ingestion with each main meal (PRO) did not reduce the prevalence of hyperglycemia (39±10%, 9±2 h/24 h; P=0.2).
Conclusion: Co-ingestion of a protein hydrolysate with each main meal does not improve glucose homeostasis over a 24 h period in longstanding type 2 diabetes patients.

Introduction

Glycemic control is the main objective in type 2 diabetes management. Both the U.K. Prospective Diabetes Study[1-3] and the Diabetes Control and Complications Trial[4] have shown that improved glycemic control effectively reduces the risk of developing micro- and macrovascular complications and cardiovascular disease. Glycemic control is generally assessed by measuring either HbA1c content or fasting blood glucose concentrations. However, these parameters provide little information on the prevalence of hyperglycemia throughout the day. Recent data applying continuous glucose monitoring systems in well controlled type 2 diabetes patients have shown that daily postprandial blood glucose excursions leading to hyperglycemia (defined as blood glucose concentrations >10 mmol/L) are more frequent than expected[5]. These postprandial blood glucose excursions represent a direct and independent risk factor for the development of cardiovascular complications in patients with type 2 diabetes[6-8]. Therefore, therapeutic strategies in the treatment of type 2 diabetes should aim to reduce postprandial hyperglycemia.

In longstanding type 2 diabetes patients, hyperglycemia is no longer accompanied by compensatory hyperinsulinemia and, as such, it is generally assumed that insulin secretory capacity of the β-cell is severely impaired. Such a defect is indicative of a progressive insensitivity of the β-cell to glucose[9]. Even though insulin secretion in response to glucose ingestion is blunted in longstanding type 2 diabetes patients, we have shown that co-ingestion of a protein hydrolysate/leucine mixture can stimulate endogenous insulin secretion resulting in a 2-4 fold greater postprandial insulin response[10-12]. This increased postprandial insulin response has been shown to accelerate blood glucose disposal, attenuating the postprandial rise in blood glucose concentrations in these type 2 diabetes patients[10]. More recently, we reported that co-ingestion of a protein hydrolysate/leucine mixture after each main meal improves 24 h blood glucose homeostasis in longstanding type 2 diabetes patients by reducing the prevalence of hyperglycemia by as much as 26%[13].

Ample evidence has been provided to support that co-ingestion of a mixture of a protein hydrolysate with free leucine represents an effective nutritional strategy to improve blood glucose homeostasis in type 2 diabetes patients. Due to the ongoing debate on the safety of free amino acids as dietary supplements[14], the addition of free amino acids to improve protein quality and/or function is either restricted or even prohibited in most countries. Therefore, in the present study, we assessed whether co-ingestion of a protein hydrolysate without additional free leucine can be used as an effective nutritional strategy to modulate 24 h blood glucose homeostasis in longstanding type 2 diabetes patients.

Subjects and methods

Subjects

13 longstanding, male type 2 diabetes patients participated in this study (age: 62±2 y; bodyweight: 87±4 kg; BMI: 28±1 kg/m^2; HbA1c: 7.8±0.3%). Exclusion criteria were impaired renal or liver function, extreme obesity (BMI>35 kg/m^2), cardiac disease, hypertension, diabetes complications, and exogenous insulin therapy. type 2 diabetes patients were using either metformin (n=2), a sulfonylurea derivative (n=2) or metformin in combination with sulfonylureas (n=9). All subjects were informed about the nature and the risks of the experimental procedures before their written informed consent was obtained. The Medical Ethical Committee of the Academic Hospital in Maastricht approved all clinical trials.

Medication, diet and activity prior to testing

Blood glucose lowering medication was withheld for 2 d prior to the screening but continued throughout the experimental trials. All subjects maintained normal dietary and physical activity patterns throughout the entire experimental period, and refrained from exhaustive physical labor and/or exercise training for at least 3 d prior to each trial. Food intake and physical activity questionnaires were collected for 2 d prior to the trials to keep dietary intake and physical activity as identical as possible. The evening before each trial, subjects received a standardized meal (see Diet & Physical activity).

Screening & Study design

After an overnight fast, all subjects performed a standard 2 h, 75 g oral glucose tolerance test (OGTT). Type 2 diabetes was confirmed according to the 2003 ADA guidelines[15].

Each subject participated in a randomized, double blind crossover design. Subjects were studied on 2 occasions for 40 h under strict dietary standardization but otherwise normal, free-living conditions with the use of a continuous glucose monitoring system (CGMS). In one trial, subjects received 3 beverages containing a protein hydrolysate (PRO), and in the other trial, a placebo beverage (PLA) was provided.

Protocol

Prior to the start of the first 40 h assessment period subjects reported to the laboratory in the afternoon and were given instructions regarding their standardized diet, the consumption of the experimental beverages and on the correct use of the food intake and physical activity questionnaires. All subjects received a short training in the use of the capillary blood sampling method (Glucocard Memory PC, A. Menarini Diagnostics, Firenze, Italy). Next, a microdialysis fiber (Medica, Medolla, Italy) was inserted in the peri-umbilical region. The micro-fiber was subsequently connected to a portable continuous

glucose-measuring device (CGMS; GlucoDay®S, A. Menarini Diagnostics, Firenze, Italy). Thereafter, subjects were provided with their diet and were allowed to return home and resumed their normal daily activities. The following day the subjects consumed their designated meals, drinks and snacks at the set time-points. Before consuming a meal, subjects obtained a capillary blood glucose sample, and after finishing the meal the subjects drank a bolus beverage containing either PRO or PLA. The subsequent day, subjects reported back to the laboratory where the CGMS was removed. CGMS data of the second day (from 07.00 to 07.00 am.) were used for data analyses.

Diet and physical activity

All meals, snacks and beverages were provided in pre-weighed packages and ingested at pre-determined time-points to ensure fully standardized dietary modulation during the two 40 h test periods. On the evening prior to the 24 h analysis period, all subjects received a standardized meal (43.8 kJ/kg bw; consisting of 60 Energy% (En%) carbohydrate, 28 En% fat and 12 En% protein). The following day, subjects consumed their meals, drinks and snacks at set time-points. The standardized diet (3 meals and 3 snacks per day) provided 12.2±0.5 MJ/d, and consisted of 64 En% carbohydrate, 25 En% fat and 11 En% protein. After consumption of each main meal, subjects ingested a pre-packaged bottle containing either PRO or PLA. Ingestion of the protein hydrolysate beverages represented an additional daily energy intake of 1728 kJ per day, and modulated the macronutrient composition of the diet to 54 En% carbohydrate, 22 En% fat and 25 En% protein. During both test periods, subjects filled out food intake and exercise questionnaires. The rate of energy expenditure during the intervention day was determined using the Compendium of Physical Activities[16] and averaged 11.7±0.8 and 11.5±0.6 MJ/d in the PLA and PRO trial, respectively (NS).

Beverages

Beverages consisted of 4 mL/kg of water (PLA) or water containing 0.4 g/kg casein protein hydrolysate (PRO) and had to be ingested directly after each meal. The casein protein hydrolysate was prepared by DSM Food Specialties (Delft, the Netherlands). The casein hydrolysate (Insuvital™) was obtained by enzymatic hydrolysis of sodium caseinate using a proprietary mix of proteases. Drinks were uniformly flavored by adding 0.2 g sucralose, 1.8 g citric acid, and 5 g cream vanilla flavor (Quest International, Naarden, the Netherlands) per liter beverage to make the taste comparable in both trials.

Continuous glucose monitoring system

The GlucoDay®S system is an ambulant continuous glucose monitoring system based on the microdialysis technique and allows continuous glucose monitoring for a period of 48 h[17]. A microdialysis fiber (Medica, Medolla, Italy)

was inserted in the peri-umbilical region, without anesthesia, using an 18-gauge Teflon catheter as a guide[18]. The micro-fiber was subsequently connected to the portable continuous glucose-measuring device (GlucoDay®S, A. Menarini Diagnostics, Firenze, Italy). The device consists of a peristaltic pump that pumps Dulbecco's solution at a rate of 10 µL/min through the microdialysis fiber. The subcutaneous interstitial fluid is taken up by the microdialysis fiber and is transported to the measuring cell. A detailed description of the device is provided elsewhere[19, 20]

Blood sample analysis

Blood (10 mL) was collected in EDTA containing tubes and centrifuged at 1,000 g and 4°C for 10 min. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at -80°C until analyses. Glucose concentrations (Uni Kit III, Roche, Basel, Switzerland) were analyzed with the COBAS FARA semi-automatic analyzer (Roche). Plasma insulin was determined by radioimmunoassay (HI-14K, Linco research Inc, St. Charles, USA). To determine HbA1c content a 3 mL blood sample was collected in EDTA containing tubes and analyzed by high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany).

Statistics and data analysis

The acquired data were downloaded from the CGMS to a personal computer with GlucoDay® software (V3.0.5). Values reported by the CGMS were converted into glucose values using the SMBG values. The efficacy and the accuracy of the GlucoDay®S has been validated for both diabetes patients[17, 21] and healthy subjects[22]. CGMS data of the second day (from 07.00 to 07.00 am.) were used for data analyses and are expressed as means±SEM. All parameters were analyzed over the entire 24 h measuring period and postprandially (i.e. 4 h after breakfast and for 6 h after lunch and dinner). To quantify and compare the prevalence of hyperglycemia between the trials, the amount of time during which glucose concentrations were >10 mmol/L was calculated. An ANOVA or a Student's t-test for paired observations was applied where applicable. Significance was set at the 0.05 level of confidence. All statistical calculations were performed using StatView 5.0 (SAS Institute inc., Cary, NC, USA).

Results

A total of 13 type 2 diabetes patients (age: 62±2 y; BMI: 28±1 kg/m^2) were recruited to participate in this study. All patients had been diagnosed with type 2 diabetes for several years (7±1 y). HbA1c content averaged 7.8±0.3%. Fasting glucose concentrations averaged 10.8±0.8 mmol/L and were significantly increased at the end of the OGTT (18.9±1.5 mmol/L; $P<0.01$). Fasting plasma insulin concentrations were within a normal range (19.6±3.6 mU/L) Whole-

body insulin sensitivity was calculated using the oral glucose insulin sensitivity (OGIS)-index[23] and averaged 238±16 mL/min/m^2.

Table 6.1. Blood glucose concentrations

	Placebo	Protein	P value
24 h	8.9±0.8	9.2±0.7	0.5
Breakfast	10.6±0.9	10.3±0.8	0.6
Lunch	9.5±1.0	10.0±0.9	0.5
Dinner	8.7±0.8	8.9±0.8	0.6

All values are expressed as means±SEM. Mean plasma glucose concentrations, expressed in mmol/L within a 24h period, and following the first 4h (after the breakfast) and 6h (after lunch and dinner) after ingestion of a main meal.

Average 24 h blood glucose concentrations are illustrated in Figure 6.1. Fasting glucose concentrations averaged 7.7±0.7 and 7.6±0.7 mmol/L in the PLA and PRO trial, respectively (P=0.9). Mean 24 h blood glucose concentration averaged 8.9±0.8 and 9.2±0.7 mmol/L in the PLA and PRO trial, respectively and did not differ between trials.

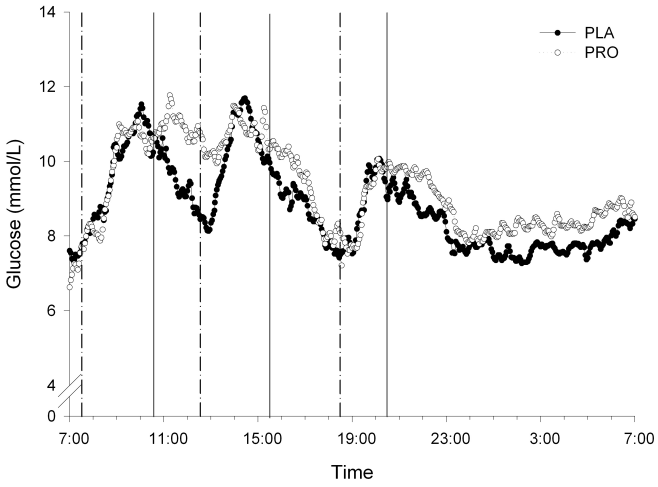

Figure 6.1. Mean plasma glucose concentrations over a 24h period while using a standardized diet with (open symbols) or without (filled symbols) co-ingestion of a protein hydrolysate with each main meal in type 2 diabetes patients. Vertical dashed lines indicate time of breakfast (07:30), lunch (12:30) and dinner (18:30), respectively. Solid lines indicate between-meal snacks.

No differences were observed in postprandial glucose concentrations between trials (Table 6.1). Although subjects continued their oral blood glucose lowering medication and consumed a healthy diet during the intervention period, hyperglycemia (here defined as blood glucose concentrations >10 mmol/L) was present 34±9% of the time (8±2 h) over the 24 h period in the PLA trial (Table

6.2). Ingestion of the PRO mixture did not modulate the prevalence of hyperglycemia (39±10%, 9±2 h; P=0.2).

Table 6.2. Hyperglycemia

	Placebo	Protein	P value
24 h	8:08′±2:24′	9:28′±2:14′	0.2
Breakfast	2:04′±0:24′	2:03′±0:23′	0.9
Lunch	2:28′±0:42′	3:01′±0:39′	0.2
Dinner	2:00′±0:40′	2:18′±0:10′	0.5

All values are expressed as means±SEM. Prevalence of hyperglycemia, expressed as the total amount of time (hh:mm) during which blood glucose concentrations were elevated above 10 mmol/L: as observed within the total 24 h period, and during each postprandial period.

Discussion

The present study shows that type 2 diabetes patients who receive standard primary medical care experience hyperglycemia throughout the larger part of the day. Co-ingestion of a protein hydrolysate with each main meal does not reduce the prevalence of hyperglycemia in these longstanding type 2 diabetes patients.

With the use of a validated continuous glucose monitoring system[17, 19-22], we assessed 24 h blood glucose concentrations in longstanding type 2 diabetes patients under strict dietary standardization but otherwise free-living conditions. Despite a healthy, balanced diet and the continued use of oral blood glucose lowering medication[24], these type 2 diabetes patients showed substantial hyperglycemia throughout the larger part of the day. The prevalence of such elevated postprandial blood glucose excursions in type 2 diabetes patients imposes a direct and independent risk for the development of cardiovascular complications[6-8]. In accordance, both the Diabetes Control and Complications Trial[4] and the U.K. Prospective Diabetes Study[1-3] reported that improving glycemic control effectively reduces the risk of developing micro- and macrovascular complications and cardiovascular disease. Therefore, therapeutic strategies in the treatment of type 2 diabetes should focus on reducing these postprandial hyperglycemic blood glucose excursions.

The blunted insulin secretory response following carbohydrate ingestion represents an important factor contributing to the elevated postprandial blood glucose excursions in these longstanding type 2 diabetes patients[12]. This reduced insulin response is attributed to the progressive insensitivity of the β-cell to glucose rather than a reduced insulin secretory capacity[12, 25]. Therefore, other insulin secretagogues like amino acids, can be applied to augment endogenous insulin release in these patients. Several groups have proposed that protein (hydrolysate) and/or amino acid ingestion forms an effective strategy to augment postprandial endogenous insulin release thereby effectively

reducing postprandial blood glucose excursions[26-28]. Both *in vivo* and *in vitro* work has identified leucine as a particularly interesting insulin secretagogue, as leucine both induces and enhances pancreatic β-cell insulin secretion and could also help to maintain β-cell mass[29-31]. As such, leucine co-ingestion has been suggested as an effective strategy to augment the insulinotropic effects of protein co-ingestion. In accordance, we established that co-ingestion of a protein hydrolysate/leucine mixture with carbohydrate can be used to augment endogenous insulin secretion, accelerate blood glucose disposal and to attenuate the postprandial rise in blood glucose concentrations in type 2 diabetes patients[10-12]. More recently, we showed that co-ingestion of such a protein/amino acid mixture with every main meal can be used as an effective nutritional intervention strategy to reduce daily postprandial hyperglycemia in these patients by ~26%[13]. There has been intense debate on the safety of free amino acids as dietary supplements[14]. As such, the addition of free amino acids (like leucine) to food(stuffs) to improve protein quality or function as metabolically active components is either restricted or even prohibited in most countries. Therefore, in the present study, we aimed to assess the practical relevance of the co-ingestion of an insulinotropic protein hydrolysate without additional free leucine as a nutritional intervention strategy to improve daily glycemic control in longstanding type 2 diabetes patients.

We confirm our previous findings[5] showing that longstanding, well controlled type 2 diabetes patients are in a state of hyperglycemia throughout a considerable part of the day (8±2 h/24 h), despite the continued use of oral blood glucose lowering medication. These findings support the current belief that pharmacological treatment with oral blood glucose lowering medication does not provide adequate protection against hyperglycemia[5, 32] and that additional strategies need to be developed to improve glycemic control in type 2 diabetes patients. In the present study, we failed to observe any modulating effect of protein hydrolysate co-ingestion on daily glycemic control. No differences in the prevalence of hyperglycemia (8±2 vs 9±2 h/24 h; P=0.2) were observed between the placebo and protein trials, respectively (Table 6.2). These data tend to be not in line with our previous findings[13], in which we reported a 26±9% decline in hyperglycemia in a similar group type 2 diabetes patients. Except for the absence of additional free leucine co-ingestion, the applied intervention and research design were identical between studies. Although the use of continuous glucose monitoring devices in an applied setting do not allow for concomitant insulin measurements, other studies[10-12, 26, 33] have repeatedLy shown that protein/amino acid co-ingestion stimulates insulin secretion resulting in enhanced glucose disposal and a reduced postprandial glucose response. We speculate that co-ingestion of free leucine is instrumental to maximize the insulinotropic response[11, 34] and, as such, to maximize the impact on glycemic control. However, it should be noted that in the present study protein hydrolysate was administered on top of the same standardized diet that

was provided in the PLA trial. So we need to stress that similar 24 blood glucose kinetics were reported in the PRO trial, despite the fact that total energy intake was ~14% greater in the PRO versus PLA trial.
In the present study, we investigated the acute response of protein hydrolysate co-ingestion on glycemic control on a daily basis. Besides the acute effects on endogenous insulin release, protein (hydrolysate) co-ingestion likely has more beneficial properties when implemented in the diet for a more prolonged intervention period. Increasing the amount of dietary protein has been suggested to increase satiety and reduce total energy intake, thereby stimulating weight loss[35]. In a prolonged intervention study, Gannon and Nuttal[36] reported improved glycemic control over a 5-week intervention after increasing the protein content in the diet. Though this substantially improved glycohemoglobin levels, it should be noted that dietary protein intake was increased at the expense of carbohydrate intake, thereby automatically lowering postprandial blood glucose excursions. More research is warranted regarding the use of protein (hydrolysates) and/or specific amino acids as pharmaconutrients in the prevention and/or treatment of type 2 diabetes.
In conclusion, longstanding type 2 diabetes patients experience hyperglycemia throughout a large part of the day, despite the use of oral blood glucose lowering medication. Co-ingestion of a protein hydrolysate after each main meal does not have a substantial effect on glycemic control in type 2 diabetes patients.

Acknowledgements

The authors would like to thank A. Menarini Diagnostics BENELUX and Hanneke van Milligen for technical assistance and the subjects who volunteered to participate in these trials for their enthusiastic support. The described work was supported by a grant from DSM Food Specialties (Delft, The Netherlands).

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Chapter 7

The muscle protein synthetic response to carbohydrate and protein ingestion is not impaired in men with longstanding type 2 diabetes

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J Nutr, 2008; 138(6): 1079-85

Abstract

Objective: To investigate whether type 2 diabetes patients show an impaired muscle protein synthetic response to food ingestion.
Design: Ten male type 2 diabetes patients using their normal oral glucose-lowering medication (68±2 y) and 10 matched, normoglycemic men (65±2 y) were randomLy assigned to 2 cross-over treatments in which whole body and muscle protein synthesis were measured following the consumption of either carbohydrate (CHO) or carbohydrate with a protein hydrolysate (CHO+PRO). Primed, continuous infusions with L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine were applied, and blood and muscle samples were collected to assess whole-body protein balance and mixed muscle protein fractional synthetic rate (FSR) over a 6 h period.
Results: Whole-body phenylalanine and tyrosine flux were significantly higher in CHO+PRO compared to CHO treatment in the diabetes and control group ($P<0.01$). Protein balance was negative following CHO, but positive following CHO+PRO treatment in both groups. Muscle protein synthesis rates were significantly higher following CHO+PRO compared to CHO treatment (0.086±0.014 vs 0.040±0.003%/h; $P<0.01$). No group differences were observed between the diabetes patients and normoglycemic controls.
Conclusion: We conclude that the muscle protein synthetic response to carbohydrate or carbohydrate plus protein ingestion is not substantially impaired in longstanding, type 2 diabetes patients treated with oral blood glucose lowering medication.

Introduction

The gradual loss of skeletal muscle mass with aging is accompanied by reduced muscle strength and function[1, 2]. As skeletal muscle tissue is responsible for up to 80% of whole-body glucose uptake, it is evident that the gradual decline in muscle mass lowers blood glucose disposal capacity. The latter represents a major factor, contributing to the development of insulin resistance and/or type 2 diabetes at a more advanced age. The loss of skeletal muscle mass is attributed to a disruption in the regulation of skeletal muscle protein synthesis and/or degradation[3]. Basal muscle protein synthesis and whole-body protein turnover rates have been shown to be similar in type 2 diabetes patients and healthy controls[4]. However, there are some indications that total whole-body protein turnover rates can be elevated in suboptimal controlled diabetes patients[5]. It has been suggested that the insulin resistant state is accompanied by a blunted muscle protein anabolic response to food intake[6, 7]. As such, the gradual loss of skeletal muscle mass with aging can be regarded both cause as well as consequence of insulin resistance and type 2 diabetes[2, 6, 8, 9], Therefore, long-term dietary and exercise interventions that stimulate muscle protein synthesis or reduce muscle protein breakdown are likely effective in the prevention and treatment of insulin resistance and/or type 2 diabetes at a more advanced age.

Protein ingestion has been shown to promote muscle protein accretion in healthy, young individuals[10]. Recent studies suggest that the muscle protein anabolic response to food intake is blunted in the elderly[11-13]. This blunted anabolic response has been attributed to skeletal muscle protein synthesis being more resistant to the stimulating effects of elevated plasma amino acid and/or circulating insulin levels[14] and might be attributed to both impairments in insulin stimulated muscle perfusion[14] and a reduced responsiveness of the mRNA translation initiation machinery[11, 15]. However, the relative contribution of each of these, alongside other unidentified aspects, is still undefined. As the muscle protein synthetic response to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly[12], we hypothesize that the anabolic response to protein ingestion is even more impaired in type 2 diabetes patients at a more advanced age.

Many studies have reported on the stimulating effect of the combined ingestion of carbohydrate and protein on postprandial insulin release *in vivo* in humans[16, 17]. In our laboratory, we have shown that co-ingestion of a protein hydrolysate with carbohydrate can be applied to improve glucose homeostasis in longstanding type 2 diabetes patients by strongly stimulating endogenous insulin release[18, 19]. Insulin has also been reported to stimulate protein synthesis under conditions of hyperaminoacidemia[20, 21] and can effectively reduce muscle proteolysis[22, 23]. Therefore, co-ingestion of an insulinotropic protein hydrolysate with carbohydrate could represent an effective strategy to stimulate the muscle protein anabolic response to food intake in longstanding, type 2 diabetes patients[24]. In the present study, we assessed the muscle protein synthetic

response to the ingestion of carbohydrate and carbohydrate plus protein in longstanding, type 2 diabetes patients under normal, practical conditions, in which blood glucose lowering medication is maintained. This is the first study to compare the muscle protein synthetic response to food intake between longstanding, type 2 diabetes patients under standard medical care and matched, normoglycemic controls.

Subjects and methods

Subjects

Ten longstanding (diagnosed with type 2 diabetes for over 5 y), male type 2 diabetes patients and 10, for age and BMI matched, normoglycemic controls were selected to participate in this study (Table 7.1). Exclusion criteria were impaired renal or liver function, extreme obesity (BMI>35 kg/m^2), cardiac disease, hypertension, diabetes complications and exogenous insulin therapy. The type 2 diabetes patients were using either metformin (n=2), a sulfonylurea derivative (n=1), metformin in combination with sulfonylureas (n=5) or dietary modulation only (n=2). All subjects were informed about the nature and the risks of the experimental procedures before their written informed consent was obtained. The Medical Ethical Committee of the Academic Hospital in Maastricht approved all clinical experiments.

Screening

All subjects performed a standard 75g oral glucose tolerance test (OGTT[6]). After an overnight fast, subjects arrived at the laboratory at 08.00 am by car or public transportation. Plasma glucose concentrations were measured to determine glucose intolerance and/or type 2 diabetes according to 2006 ADA guidelines[25]. Plasma glucose and insulin concentrations obtained during the OGTT were used to assess whole-body insulin resistance/sensitivity using the homeostasis model assessment insulin resistance index (HOMA-IR)[26] and the oral glucose insulin sensitivity (OGIS)-index[27]. Furthermore, blood HbA1c contents were determined in basal blood samples (Table 7.1).

Table 7.1. Subjects' characteristics

	Controls	Type 2 diabetes
n	10	10
Age (y)	65.3±1.5	67.8±1.8
Body weight (kg)	75.6±1.6	73.3±1.4
Height (m)	1.72±0.02	1.72±0.02
BMI (kg/m^2)	25.6±0.8	24.9±0.4
Basal plasma glucose (mmol/L)	5.6±0.1	9.7±0.9[a]
Plasma glucose$_{OGTT120}$ (mmol/L)[2]	6.3±0.3	17.4±1.5[a,b]
Basal plasma insulin (pmol/L)	99.9±8.0	93.4±7.2
HOMA-IR	3.5±0.3	5.3±0.4[a]
HbA1c (%)	5.7±0.1	7.7±0.4[a]
OGIS (mL/min·m^{-2})	388±15	265±14[a]
Diagnosed with type 2 diabetes (y)	NA	9±2
Medication	NA	Metformin and/or SU-derivatives

All values are expressed as means±SEM. [2] Plasma glucose concentration 2 h after ingesting 75 g glucose. [a] significantly different from control group, P<0.05 (*t*-test comparing patient and control group). [b] significantly different from basal values, P<0.01 (*t*-test comparing pre and post OGTT values).

Medication, diet and activity prior to testing

Blood glucose lowering medication was withheld for 2 days prior to the screening but continued throughout the experiments. All subjects maintained normal dietary and physical activity patterns throughout the entire experimental period. Subjects refrained from heavy physical labor and/or exercise training for at least 3 days prior to each experiment and filled out a food intake questionnaire for 2 days prior to the first experiment to keep their dietary intake as identical as possible prior to the other experiment. The evening before each experiment, subjects received a standardized meal containing 44 kJ/kg bodyweight (BW); consisting of 60 energy% (En%) carbohydrate, 28 En% fat and 12 En% protein.

Experiments

Each subject participated in a randomized, double blind cross-over design. All subjects were studied on 2 occasions, separated by 7 days, in which drinks containing carbohydrate (CHO) or carbohydrate plus protein hydrolysate (CHO+PRO) were administered. Each experiment lasted ~8 h. Repeated boluses of a given test-drink were administered to ensure a continuous supply of glucose and amino acids from the gut throughout the experiment. Plasma and muscle samples were collected during a 6 h measurement period. These experiments were designed to simultaneously assess whole-body amino acid

kinetics and mixed muscle protein fractional synthetic rate (FSR) in the *m. vastus lateralis*.

Protocol

At 8.00 am, after an overnight fast, subjects arrived at the laboratory by car or public transportation. Teflon catheters were inserted into an antecubital vein for stable isotope infusion and in a dorsal hand vein of the contra-lateral arm which was placed in a hot-box (60°C) for arterialized blood sampling. After baseline blood sample collection, a single intravenous dose of L-[ring-$^{13}C_6$] phenylalanine (2 μmol/kg) and L-[ring-$^{2}H_2$] tyrosine (0.775 μmol/kg) was administered. Thereafter, continuous tracer infusion (0.049±0.0004 μmol/kg·min^{-1} for L-[ring-$^{13}C_6$] phenylalanine and 0.018±0.0002 μmol/kg·min^{-1} for L-[ring-$^{2}H_2$] tyrosine) was started. We chose the use of L-[ring-$^{13}C_6$] phenylalanine as opposed to L-[1-^{13}C] leucine as amino tracer to study protein metabolism, as previous work[28] has shown that total amino acid oxidation rates are likely to be overestimated when using leucine as tracer. After a 2 h pre-infusion period (t=0 min), an arterialized blood sample and a muscle biopsy from the *m. vastus lateralis* were collected. Subjects then received an initial bolus (1.5 mL/kg) of a given test drink. Repeated boluses (1.5 mL/kg) were ingested every 30 min until t=330 min. Arterialized blood samples were collected at t= 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min with a second muscle biopsy taken at t=360 min from the contra-lateral leg. Subjects remained in a resting, supine position throughout the entire experiment.

Beverages

Subjects received 12 beverages with a volume of 1.5 mL/kg every 30 min over a 6 h period to ensure a given dose of 0.6 g carbohydrate/kg (50% glucose and 50% maltodextrin) with or without the addition of 0.3 g/kg of a casein protein hydrolysate per hour. In total, all subjects were provided with ~1.3 L of water, ~268 g carbohydrate and ~134 g protein hydrolysate. Repeated boluses were administered to ensure a continuous supply of amino acids in the circulation, preventing perturbations in L-[ring-$^{13}C_6$] phenylalanine and L-[ring-$^{2}H_2$] tyrosine enrichments. Glucose and maltodextrin were obtained from AVEBE (Veendam, The Netherlands). The casein protein hydrolysate was prepared by DSM Food Specialties (Delft, the Netherlands) and was obtained by enzymatic hydrolysis of sodium caseinate using a proprietary mix of proteases. Drinks were uniformly flavored by adding 0.2 g sodiumsaccharinate, 1.8 g citric acid, and 5 g cream vanilla flavor (Quest International, Naarden, the Netherlands) per liter beverage.

Analysis

Blood samples were collected in EDTA containing tubes and centrifuged at 1,000 *g* and 4°C for 10 min. Aliquots of plasma were frozen in liquid nitrogen

and stored at –80°C. Plasma glucose concentrations (Uni Kit III, 07367204, Roche, Basel, Switzerland) were analyzed with the COBAS-FARA semi-automatic analyzer (Roche). To determine HbA1c content a 3 mL blood sample was collected in EDTA containing tubes and analyzed by high-performance liquid chromatography (Bio-Rad Variant II, Munich, Germany). Insulin was analyzed by radio immunoassay (HI-14K, Linco research Inc, St. Charles, USA). Plasma (500 µL) was deproteinized with 5-sulphosalicylic acid for determination of amino acid concentrations as described previously[29]. The exact phenylalanine and tyrosine concentrations in the infusates averaged 4.66±0.04 and 1.73±0.02 mmol/L, respectively. Plasma phenylalanine and tyrosine were derivatized to their t-butyldimethylsilyl (TBDMS) derivatives and their ^{13}C and/or ^{2}H enrichments were determined by electron ionization gas chromatography-mass spectrometry (GC-MS, Agilent 6890N GC/5973N MSD Little Falls, DE, USA) as described elsewhere [21].

For measurement of L-[ring-$^{13}C_6$] phenylalanine enrichment in both the free amino acid and mixed muscle protein pool, 55 mg of wet muscle was freeze-dried and processed as described previously[21]. The free amino acid concentrations in the muscle supernatant were measured by HPLC, after precolumn derivatization with *o*-phthaldialdehyde[30], whereas intracellular free L-[ring-$^{13}C_6$] phenylalanine, L-[ring-$^{2}H_2$] tyrosine and L-[ring-$^{13}C_6$] tyrosine enrichments were measured using their TBDMS derivatives on a GC-MS[21]. Muscle bound phenylalanine enrichment was determined using its *N(O,S)*-ethoxycarbonyl ethyl ester for the determination of ^{13}C:^{12}C ratios on a GC-IRMS[31].

Calculations

Infusion of L-[ring-$^{13}C_6$] phenylalanine and L-[ring-$^{2}H_2$] tyrosine with muscle and arterialized blood sampling was used to simultaneously assess whole-body amino acid kinetics and fractional synthetic rate (FSR) of mixed muscle protein. Whole-body kinetics for phenylalanine and tyrosine were calculated based on the equations described by Short et al.[32]. Fractional rate of mixed muscle protein synthesis (FSR) was calculated by dividing the increment in enrichment in the product, i.e. protein-bound L-[ring-$^{13}C_6$] phenylalanine, by the enrichment of the precursor (plasma L-[ring-$^{13}C_6$] phenylalanine enrichment) as described previously[21].

Statistics

All data are expressed as means ± SEM. A paired student t-test was applied for the analysis of pre and post OGTT values. Plasma essential amino acid, insulin and glucose responses were calculated as area under the curve above baseline values. A 3-factor repeated measure ANOVA with group, time and treatment as factors was used to compare differences between treatments over time between groups. For non-time dependent variables, a 2-factor ANOVA with group and

treatment as factors was used to compare differences in treatment effects between groups. In case of significant difference between treatments, Scheffe's post-hoc test was applied to locate these differences. Paired student's t-tests were used to compare fasting and 2 h OGTT values. Statistical significance was set at $P<0.05$. All calculations were performed using StatView 5.0 (SAS Institute inc., Cary, NC, USA).

Results

Plasma analyses

Fasting plasma insulin concentrations during the experiments did not differ between the type 2 diabetes patients and normoglycemic controls (79±7 vs 98±12 pmol/L, respectively; P=0.19). Overall, plasma insulin responses, expressed as area under the curve above baseline values (AUC), were higher in CHO+PRO vs CHO treatment (Table 7.2; estimated marginal means: 176±24 vs 125±14 nmol/6h·L^{-1}, respectively; $P<0.05$).

Table 7.2. Plasma insulin and glucose responses following carbohydrate or carbohydrate plus protein ingestion in type 2 diabetes patients and normoglycemic control subjects

	Controls		Type 2 diabetes		P values		
	CHO	CHO+PRO	CHO	CHO+PRO	Group	Treat-ment	Inter-action
Insulin (nmol/L·6h^{-1})	169±20	198±26	80±20	155±39	0.08	<0.01	0.16
Glucose (mol/L·6h^{-1})	1.0±0.1	0.6±0.12[a]	2.9±0.33[b]	2.0±0.22[a,b]	<0.01	<0.01	0.01

All values are expressed as means±SEM. n=10 normoglycemic controls and n=10 type 2 diabetes patients
[a] significantly different from the CHO experiment (Scheffe's test, P<0.05).
[b] significantly different from values observed in the control group (Scheffe's test, P<0.05).

Fasting plasma glucose concentrations were higher in the diabetes patients compared with the controls (9.3±1.0 and 5.7±0.1 mmol/L, respectively P<0.01). In the diabetes and control groups, plasma glucose responses were 32±8 and 32±9% lower after the CHO+PRO trial than after the CHO trial respectively (P<0.05; Table 7.2). No differences were found in basal plasma phenylalanine, tyrosine or branched chain amino acid (leucine, isoleucine and valine) concentrations between groups. A complete overview of the subsequent plasma amino acid responses is provided in Table 7.3.

Table 7.3. Plasma amino acid responses following the ingestion of carbohydrate or carbohydrate plus protein in type 2 diabetes patients and normoglycemic control subjects

	Controls		Type 2 diabetes		P values		
mmol/6h·L^{-1}	CHO	CHO+PRO	CHO	CHO+PRO	Group	Treat-ment	Inter-action
Isoleucine	-5.3±1.0	35.0±2.3[a]	-4.4±0.8	41.5±2.0[a]	0.24	<0.01	0.14
Leucine	-9.1±1.2	56.3±3.9[a]	-6.4±1.0[b]	69.5±2.7[a,b]	0.01	<0.01	0.05
Phenylalanine	1.4±0.7	20.0±1.5[a]	1.7±0.5	19.5±0.9[a]	0.93	<0.01	0.73
Tyrosine	-1.5±0.8	35.4±2.4[a]	-0.8±0.4	35.9±1.8[a]	0.74	<0.01	0.98
Valine	-6.2±1.7	87.7±5.2[a]	-4.2±1.0[b]	99.3±3.8[a,b]	0.04	<0.01	0.20

All values are expressed as means±SEM. n=10 normoglycemic controls and n=10 type 2 diabetes patients.
[a] significantly different from the CHO experiment (Scheffe's test, P<0.05). [b] significantly different from values observed in the control group (Scheffe's test, P<0.05).

Muscle analyses

No differences in free leucine, valine, isoleucine, phenylalanine and tyrosine concentrations were observed in the muscle biopsies taken at t=0 min between the type 2 diabetes patients and control subjects. At t=360 min, muscle free leucine and valine concentrations were significantly higher in the CHO+PRO compared with the CHO treatment (estimated marginal means, leucine: 222±14 vs 127±11 μmol/L, and valine: 241±17 vs 134±13 μmol/L, respectively, P<0.05), with no significant differences between groups. Muscle free isoleucine concentrations at t=360 min were significantly higher in the CHO+PRO compared with the CHO treatment in the control group (138±11 vs 57±10 μmol/L, respectively, P<0.05), but not in the diabetes patients (95±11 vs 109±10 μmol/L, respectively). Muscle free phenylalanine concentrations at t=360 min were significantly higher in the CHO+PRO compared with the CHO treatment in the control group (106±8 vs 62±4 μmol/L, respectively, P<0.05), but were not significantly different between the CHO and CHO+PRO treatment in the diabetes patients (93±7 vs 81±6 μmol/L, respectively). Muscle free tyrosine concentrations at t=360 min were significantly higher (P<0.05) in the CHO+PRO compared with the CHO treatment in the diabetes patients (159±9 vs 60.2±5 μmol/L, respectively) and control group (132±14 vs 81±8 μmol/L, respectively). Mean plasma amino acid enrichments during the last 4 h of the experiment, muscle free amino acid enrichments in the 6 h muscle biopsy and the increments in muscle protein enrichment are presented in Table 7.4. In the muscle biopsies collected at 6 h, free L-[ring-$^{13}C_6$] phenylalanine, L-[ring-$^{2}H_2$] tyrosine and L-[ring-$^{13}C_6$] tyrosine enrichments were not different between the type 2 diabetes patients and normoglycemic controls. Overall, free L-[ring-$^{13}C_6$] phenylalanine, L-[ring-$^{2}H_2$] tyrosine, and L-[ring-$^{13}C_6$] tyrosine enrichments in the 6 h muscle biopsy were significantly higher in the CHO compared with the

CHO+PRO treatment (P<0.05). No differences were observed in the increase in muscle protein bound L-[ring-$^{13}C_6$] phenylalanine enrichment in the CHO and CHO+PRO treatments between the type 2 diabetes patients and control group.

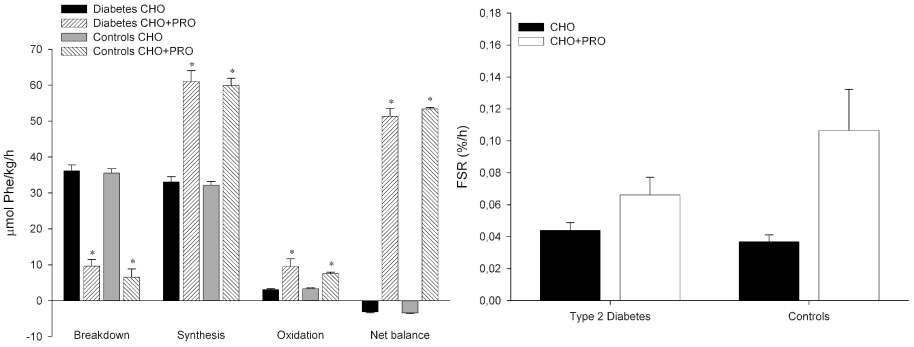

Figure 7.1. Panel A: Rate of whole-body protein breakdown, synthesis, oxidation and net protein balance following carbohydrate or carbohydrate plus protein ingestion in type 2 diabetes patients and normoglycemic controls. Values are means±SEM. Data were analyzed with ANOVA (group x treatment). Breakdown: group effect, P=0.38; treatment effect, P<0.01; interaction of group and treatment, P=0.41. Synthesis: group effect, P=0.66; treatment effect, P<0.001; interaction of group and treatment, P=0.95. Oxidation: group effect, P=0.47; treatment effect, P<0.01; interaction of group and treatment, P=0.29. Net balance: group effect, P=0.47; treatment effect, P<0.01; interaction of group and treatment, P= 0.29. Panel B: Fractional synthetic rate of mixed muscle protein following carbohydrate or carbohydrate plus protein ingestion in type 2 diabetes patients and normoglycemic controls. Group effect, P=0.45; treatment effect, P<0.05; interaction of group and treatment, P=0.21.

Whole-body protein metabolism

Overall, phenylalanine flux was higher in the CHO+PRO compared with the CHO treatment (69.±1.3 vs 35.9±1.3 µmol phe/kg·h^{-1}, respectively, P<0.01). No differences in whole-body phenylalanine flux were found between the diabetes patients and normoglycemic controls. Whole-body protein breakdown (Figure 7.1A), was lower in the CHO+PRO compared with the CHO treatment (P<0.01). Whole-body protein synthesis was higher in the CHO+PRO compared with the CHO treatment (P<0.01). The rate of whole-body phenylalanine oxidation was higher in the CHO+PRO compared with the CHO treatment (P<0.05). Whole-body protein balance was negative in the CHO treatment, whereas protein balance was positive in the CHO+PRO treatment. Whole-body protein breakdown, synthesis, oxidation rates and net protein balance did not differ between the type 2 diabetes patients and normoglycemic controls.

Mixed muscle protein synthesis rates

Mixed muscle protein fractional synthetic rates (FSR), with the plasma L-[ring-$^{13}C_6$] phenylalanine enrichment as precursor, are shown in Figure 7.1B. Overall, mixed-muscle protein synthesis rates were significantly higher in the CHO+PRO compared to the CHO treatment (0.086±0.014 vs 0.040±0.003%/h; P<0.01). No differences were observed between groups.

Table 7.4. Tracer enrichments following the ingestion of carbohydrate or carbohydrate plus protein in type 2 diabetes patients and normoglycemic controls

	Experiment		P values [2]		
	CHO	CHO+PRO	Group	Treat-ment	Inter-action
Plasma AA enrichment (TTR) [4]					
L-[ring-$^{13}C_6$] phenylalanine			0.501	<0.001	0.576
Control	0.104±0.003	0.057±0.001			
Type 2 diabetes	0.103±0.004	0.053±0.002			
L-[ring-$^{2}H_2$] tyrosine			0.241	<0.001	<0.001
Control	0.042±0.002	0.021±0.001 [a]			
Type 2 diabetes	0.052±0.002[b]	0.020±0.001 [a]			
L-[ring-$^{13}C_6$] tyrosine			0.236	<0.001	0.254
Control	0.012±0.001	0.004±0.001			
Type 2 diabetes	0.011±0.001	0.003±0.001			
Muscle AA enrichment (TTR) [5]					
L-[ring-$^{13}C_6$] phenylalanine			0.671	<0.001	0.653
Control	0.060±0.004	0.046±0.002			
Type 2 diabetes	0.063±0.005	0.046±0.001			
L-[ring-$^{2}H_2$] tyrosine			0.384	<0.05	0.399
Control	0.037±0.003	0.016±0.001			
Type 2 diabetes	0.033±0.004	0.016±0.002			
L-[ring-$^{13}C_6$] tyrosine			0.205	0.070	<0.05
Control	0.028±0.005	0.005±0.001			
Type 2 diabetes	0.017±0.003	0.007±0.002			
Δ enrichment muscle protein (TTR) [6]					
L-[ring-$^{13}C_6$] phenylalanine			0.359	0.463	0.06
Control	0.00023±0.000026	0.00036±0.000079			
Type 2 diabetes	0.00027±0.000034	0.00021±0.000037			

Values are means±SEM. n=10 normoglycemic controls and n=10 type 2 diabetes patients. [2] Data were analyzed with ANOVA (group x treatment). [4] Mean plasma amino acid (AA) enrichments during the last 4 h of the tests [5] muscle free amino acid enrichments in the 6 h biopsy. [6] difference between muscle protein bound phenylalanine enrichment in muscle biopsy samples taken at t=0 and 6 h. [a] significantly different from CHO experiment (ANOVA, P<0.05). [b] significantly different from control group (ANOVA, P<0.05).

Discussion

The present study shows that co-ingestion of protein with carbohydrate improves whole-body protein balance and augments mixed muscle protein synthesis rates in longstanding type 2 diabetes patients and matched, normoglycemic controls.

Insulin resistance and type 2 diabetes are characterized by impairments in glucose and fat metabolism[25]. In addition, impairments in insulin sensitivity may also extend to protein metabolism in the insulin resistant and/or type 2 diabetes state[7, 33]. However, in the fasted state, whole-body leucine[4, 34-36], phenylalanine[4, 37] and tyrosine fluxes[4], as well as leucine oxidation[4, 34, 35] and phenylalanine hydroxylation rates[4] do not appear different in type 2 diabetes patients when compared to matched, normoglycemic controls. Moreover, fasting mixed muscle protein synthesis rates[4] and net muscle protein balance[33] also do not seem to be altered in type 2 diabetes patients. Therefore, basal fasting protein metabolism does not appear substantially impaired in the type 2 diabetic state.

The development of glucose intolerance, insulin resistance and/or type 2 diabetes at an advanced age is generally associated with a substantial loss of skeletal muscle mass. As skeletal muscle tissue is responsible for up to 80% of whole-body glucose uptake, it is evident that the gradual decline in muscle mass lowers blood glucose disposal capacity. As the basal muscle protein turnover rates do not seem to be affected by either age[38] or the presence of the insulin resistant state, there has been an increasing interest in the impact of aging and insulin resistance on the muscle protein synthetic response to major anabolic stimuli (i.e. food intake and physical activity). The gradual loss of muscle mass with aging is believed to be attributed to a more blunted muscle protein synthetic response to food intake[11-13]. The latter has been suggested to be due to a reduced sensitivity of muscle protein synthesis to the stimulating effects of elevated plasma amino acid and/or insulin concentrations[14]. As the muscle protein synthetic response to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly[12], we hypothesized that the muscle protein synthetic response to food intake is even further reduced in longstanding, type 2 diabetes patients at a more advanced age.

In the present study, we assessed the impact of ingesting either carbohydrate or carbohydrate plus protein (hydrolysate) on whole-body protein turnover and muscle protein synthesis rates in longstanding, type 2 diabetes patients and healthy, matched controls at a more advanced age. The anabolic response to carbohydrate and/or protein ingestion did not differ between groups (Table 7.4, Figure 7.1B). In line with previous findings[4, 35], we did not detect differences in basal plasma and/or muscle free amino acid concentrations between type 2 diabetes patients and healthy, matched controls. In addition, we observed no differences in the rate of phenylalanine hydroxylation between groups

following the ingestion of carbohydrate only (Figure 7.1A). Whole-body net protein balance was shown to remain negative when only carbohydrate was ingested (Figure 7.1A). The latter is in accordance with previous observations in healthy subjects showing net protein balance to remain negative[21, 24, 39] in the absence of protein and/or amino acid co-ingestion[21, 24, 40, 41].

Administration of protein (hydrolysate) and/or amino acids with carbohydrate rapidLy increases muscle protein synthesis in both the young and elderly[21, 42]. The stimulating effect of protein/amino acid administration can be attributed to the function of amino acids as building blocks for *de novo* protein synthesis[10], the potential of amino acids to stimulate insulin secretion[19, 43], and the property of amino acids to directly stimulate protein synthesis by activating the mRNA translational machinery[44]. As such, ingestion of a mixture containing both carbohydrate and protein represents an effective nutritional intervention to stimulate the muscle protein synthetic response to food ingestion in type 2 diabetes patients. Co-ingestion of protein significantly suppressed whole-body protein breakdown, and increased protein synthesis rates in both the type 2 diabetes patients and normoglycemic controls, with no apparent differences between groups (Figure 7.1A). Co-ingestion of the protein hydrolysate increased whole-body protein synthesis rates by ~85%, when compared with the ingestion of carbohydrate only. As a result, whole-body protein net balance became positive in the CHO+PRO treatment (Figure 7.1A), with no apparent differences between groups. The latter underlines the fact that protein co-ingestion is essential for net muscle protein accretion to occur. In line with the whole-body estimates of muscle protein synthesis, skeletal muscle protein synthesis rates in the *vastus lateralis* muscle were higher following protein co-ingestion in both the type 2 diabetes patients and normoglycemic controls (Figure 7.1B). As no apparent differences in the muscle protein synthetic response to carbohydrate and/or protein ingestion were observed between groups, we conclude that the muscle protein synthetic response to carbohydrate and carbohydrate plus protein administration is preserved in longstanding type 2 diabetes patients under standard medical care, i.e. while using oral blood glucose lowering medication. The latter condition was specifically selected to allow a comparison between healthy, normoglycemic men and type 2 diabetes patients under normal, practical conditions in which these patients generally consume their diet. Despite their medication, the type 2 diabetes patients who participated in the present study showed substantially higher basal plasma glucose concentrations, HbA1c contents, and HOMA-IR values when compared with the normoglycemic controls (Table 7.1). In addition, the insulin response following the intake of carbohydrate with or without protein seemed to be blunted in the type 2 diabetes patients. The latter is not surprising as longstanding, type 2 diabetes patients were selected, and in whom compensatory hyperglycemia is no longer apparent. In accordance, the

accompanying plasma glucose responses were substantially greater in the diabetes patients when compared with the controls (Table 7.2).

Although glucose and fat metabolism are impaired in the insulin resistant state, our data imply that the muscle protein synthetic response to food intake is largely unaffected in these type 2 diabetes patients. The latter seems to be in contrast with previous reports indicating that insulin resistance and impaired myocellular signaling are key-factors in the etiology of muscle loss in the elderly[12, 14, 15]. However, our data are in line with Bell *et al*.[33] showing that the muscle protein synthetic response during a high energy-hyperinsulinemic clamp is preserved in poorly controlled, type 2 diabetes patients. The apparent discrepancy in the literature is likely attributed to the differences in the amount of nutrients that are administered[45, 46] and the medication that is being prescribed in the selected patients. In the present study, we aimed to assess the maximally stimulated muscle protein synthetic response to food intake. Therefore, we provided subjects with relative large amounts of carbohydrate (0.6 g/kg·h^{-1}) and protein (0.3 g/kg·h^{-1}) during the 6 h test-period. Future studies investigating the muscle protein synthetic response to the ingestion of smaller, meal-like, amounts of carbohydrate, fat and/or protein are warranted in various diabetes subpopulations, under different pharmaceutical treatments.

In conclusion, co-ingestion of a protein hydrolysate with carbohydrate improves whole-body protein balance and augments muscle protein synthesis rate to a similar extent in longstanding, type 2 diabetes patients and normoglycemic controls. The skeletal muscle protein synthetic response to carbohydrate and/or protein ingestion is not substantially impaired in longstanding, type 2 diabetes patients treated with oral blood glucose lowering medication.

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Chapter 8

General discussion

Type 2 diabetes has evolved from a chronic metabolic disease that mainly affected the elderly into one of the biggest health threats our society is currently facing, striking people of all ages and ethnicities[1, 2]. Apart from the traditional pharmacological approach to normalize glycemic control, several large-scale lifestyle studies[3-7] have shown that dietary modulation and increased physical activity are equally, if not more, effective in improving glycemic control in patients with type 2 diabetes. In this thesis we focussed on the potential benefits of protein (hydrolysate) and/or amino acid co-ingestion as a nutritional intervention strategy to stimulate endogenous insulin secretion and, as such, to improve glycemic control in type 2 diabetes patients. This chapter combines the results described in this thesis and elaborates on the primary findings, placing them in a broader perspective and discusses their clinical implication in the treatment of type 2 diabetes. Consequently, important issues that need to be addressed in future research will be defined.

Postprandial hyperglycemia

Normalizing plasma glucose concentrations in patients with (either type 1 or type 2) diabetes has always been the main target of treatment as disturbances in glucose homeostasis (and mainly hyperglycemia) are related to a multitude of diabetes related complications. Recently it has come to light that postprandial hyperglycemia forms both a direct and independent risk factor for cardiovascular disease in type 2 diabetes[8, 9]. If we consider the fact that most of us consume three meals each day and several between-meal snacks, it is obvious that we are in a postprandial state during the greater part of the day. Therefore, the postprandial period is the major determinant of 24 h glycemia. As a consequence, attenuating the postprandial rise in blood glucose concentrations should be considered as a main therapeutic target in the treatment of type 2 diabetes. However, the current standard measures of glycemic control (i.e. fasting blood glucose concentrations and hemoglobin A1c) provide insufficient insight in the prevalence of postprandial glucose fluctuations.

With recent developments in biosensor technology, continuous glucose monitoring devices have been developed which can be applied to provide more accurate insight in the prevalence of hyperglycemia throughout the day. We applied such a system to assess glycemic control in longstanding type 2 diabetes patients under standardized dietary, but otherwise free living conditions. As described in chapter 4, we observed that under standardized dietary but otherwise free living conditions these patients were in a state of hyperglycemia for more than 13 hours during the day, despite their continued use of oral blood glucose lowering medication. Even patients with a HbA1c level below 7% showed to be hyperglycemic for as much as 11 h per day. In a follow-up study (chapter 6) we confirmed that hyperglycemia is prevalent

during the greater part of the day in well-controlled type 2 diabetes patients. The findings clearly showed that current measures of glycemic control do not provide accurate insight into the prevalence of postprandial glucose fluctuations. These findings are in line with previous work by others[10-12] and imply that the current pharmacological strategies to improve glucose homeostasis with oral blood glucose lowering medication provide inadequate protection against (postprandial) hyperglycemia.

Therefore, dietary and/or physical activity interventions should be applied to complement pharmaceutical strategies to improve glycemic control in type 2 diabetes. One of the dietary strategies to reduce postprandial glycemia would be to reduce the carbohydrate content of the diet. However, there are strong arguments against the use of diets low in carbohydrate, especially considering the fact that this generally leads to the ingestion of high-fat foods, which inadvertently can lead to weight gain and the development of insulin resistance[15, 16]. Furthermore, carbohydrate containing foods are an important source of micronutrients and fiber and, as such, form an intricate part of a healthy diet.

In the 1980s the concept of the glycemic index of food and food products was suggested as a potential tool in the management of blood glucose homeostasis [16-24]. Although the principle of low GI food stuffs works in theory, the efficiency of low glycemic index diets to improve glycemic control remains debatable [25-32]. The apparent contradictory findings on the proposed health benefits of low glycemic diet are, at least partly, attributed to the fact that glycemic index (or the more frequently used glycemic load) of a specific food is calculated based on the glycemic response to the ingestion of this specific food. This does not take into account the interaction between different food products or food components in a mixed meal on the postprandial glycemic response. Furthermore, differing methods of food preparation can yield very different glycemic indices from the same food.

One of the factors that can strongly modulate the glycemic response to the ingestion of carbohydrate containing foods is the protein content of a meal. This modulating factor is attributed to the strong insulinotropic properties of amino acids.

Amino acid induced insulin secretion

The insulinotropic properties of proteins and free amino acids have been known since the 1960s[33-35] and have been well documented in both normoglycemic[36, 37] and diabetic[38-41] populations. Partly due to its insulinotropic potential, protein is now often used in post-exercise recovery sports drinks to maximize muscle glycogen[42] and protein[43] synthesis after exercise. However, the insulin stimulating properties of protein could also be of more clinical benefit to stimulate endogenous insulin release in type 2 diabetes patients. The

latter could represent an interesting strategy to stimulate postprandial glucose disposal and, as such, to attenuate the postprandial rise in blood glucose concentrations.

In longstanding type 2 diabetes patients, hyperglycemia is no longer accompanied by hyperinsulinemia. In accordance, the ingestion of carbohydrate in these patients is generally accompanied by a severely blunted insulin secretory response [39]. The inability of the pancreas to secrete sufficient amounts of insulin after carbohydrate stimulation has been related to β-cell deficiencies or even failure to produce and secrete insulin[44]. We and others hypothesized that the blunted insulin secretory response to carbohydrate ingestion is due to impairments in the glucose sensing and signaling pathway and not necessarily due to a substantial reduction in the insulin secretory capacity of the pancreas. In accordance, we showed in chapter 2 that co-ingestion of protein and amino acid mixture with carbohydrate can strongly increase the insulin response to carbohydrate ingestion in both healthy subjects and type 2 diabetes patients. As the β-cells of these patients were shown to still be able to produce large amounts of insulin, it is likely that the blunted insulin secretory response to carbohydrate is the result of glucose sensing or signal transduction defects in the β-cell to glucose alone, instead of a general failure in β-cell function. To evaluate whether the greater endogenous insulin secretory response can be of clinical relevance, we also measured plasma glucose disposal following the ingestion of carbohydrate with or without the insulinotropic protein hydrolysate/amino acid mixture. Using labeled glucose tracers, we were able to show that plasma glucose disposal was impaired in the type 2 diabetes patients compared to the normoglycemic controls. However, co-ingestion of the insulinotropic protein hydrolysate/amino acid mixture increased plasma glucose disposal and resulted in a lower postprandial glycemic response. Although the maximal rate of glucose uptake from the circulations did not seem to differ between the type 2 diabetes patients and the control subjects the time to reach this maximal uptake was strikingly different. This impairment in the ability to quickly increase glucose uptake rates in indicative of skeletal muscle insulin resistance and is probably related to defects in the GLUT-4 signaling cascade

Several *in vivo* studies show strong insulinotropic responses after administration of various free amino acids[33, 37, 46-48]. Leucine has been identified as a particular interesting insulin secretagogue, as leucine both induces and enhances pancreatic β-cell insulin secretion through its oxidative decarboxylation and its ability to allosterically activate glutamate dehydrogenase[49-52]. In order to assess the possible additional effect of leucine administration we determined the postprandial plasma insulin and glucose responses following co-ingestion of a protein hydrolysate with and without additional leucine together with a single, meal-like, bolus of carbohydrate in longstanding type 2 diabetes patients and healthy controls in chapter 3.

Although the addition of free leucine increased insulin secretion even further, postprandial glucose responses were improved similarly in both protein experiments. These data extended on previous findings[41, 53, 54], and imply that nutritional interventions with protein and leucine co-ingestion could represent an effective strategy to reduce postprandial blood glucose excursions following the ingestion of a meal-like bolus of carbohydrate.

Glycemic control through nutritional modulation

Although the insulinotropic properties of protein and amino acids when co-ingested with carbohydrate containing drinks and/or mixed meals[53] have been well described under standardized laboratory conditions, it remains to be determined whether protein and/or amino acid co-ingestion can be used as an effective and feasible therapeutic strategy under normal, free living conditions. In chapter 5 we have shown that protein and leucine co-ingestion effectively reduce the prevalence of 24 h hyperglycemia by improving postprandial glycemic control in patients with type 2 diabetes. However, it remains to be questioned whether such a nutritional intervention represents a feasible long-term strategy to improve glycemic control in type 2 diabetes patients.

In chapter 5, we observed a substantial reduction in hyperglycemia when protein and leucine were co-ingested with each main meal. These improvements in hyperglycemia were observed despite the fact that protein and leucine were added on top of the standardized diet, resulting in a higher total energy intake over the 24 h period. It is obvious that the latter does not represent a feasible long-term interventional strategy. In a long-term intervention, protein and leucine supplementation would eventually lead to changes in the macronutrient composition of an *ad libitum* diet. The greater protein intake would be accompanied by a reduction in total dietary fat and/or carbohydrate consumption. Of course, this dietary change should result in further improvements in glycemic control. In accordance, Gannon and Nuttall showed that increasing the protein content of the diet, at the expense of carbohydrate and fat, drastically lowered blood glucose concentrations in a group type 2 diabetes patients over a 5 week intervention period[40, 55, 56]. Furthermore, it should be noted that diets high in protein have been reported to be more effective when trying to maintain body weight after a period of weight loss when compared to high carbohydrate diets. This benefit has been attributed to the thermogenic and satiating properties of dietary protein[57-61].

Increasing the protein content of the diet is therefore a likely candidate to obtain desired levels of glycemic control as it has endocrine functions but could also lead to changes in body composition through the preservation of lean body mass en a reduced energy intake.

From a quantitative point of view, skeletal muscle tissue forms the most important organ responsible for blood glucose disposal. Therefore, the loss of

skeletal muscle mass with inactivity and aging represents an important contributing factor in the development of insulin resistance and/or type 2 diabetes. It has been suggested that the loss of muscle mass with aging is associated with a blunted muscle protein synthetic response to protein ingestion. As impairments in carbohydrate, fat, and protein metabolism have been reported in type 2 diabetes patients, we speculated whether the muscle protein synthetic response to carbohydrate and carbohydrate plus protein would be impaired in type 2 diabetes patients when compared to age-matched sedentary controls. Therefore, in chapter 7 we assessed the protein synthetic response to the co-ingestion of protein with carbohydrate in type 2 diabetes patients at a more advance age and matched normoglycemic controls. In contrast to previous reports in uncontrolled type 2 diabetes patients[66, 67], we observed no substantial impairments in the muscle protein synthetic response to carbohydrate or carbohydrate plus protein ingestion in the type 2 diabetic state. Co-ingestion of protein with carbohydrate improved whole body protein balance and stimulated skeletal protein synthesis to a similar extend in the diabetes patients and normoglycemic controls. As such, we concluded that the muscle protein synthetic response to food intake seems largely intact in type 2 diabetes patients and, as such, can be applied as a dietary strategy to support net muscle protein accretion and therefore prevent the decrease in glucose disposal capacity . Overall, increasing protein intake in the diet seems to represent an effective nutritional strategy to improve whole-body glycemic control in type 2 diabetes patients. However, long-term dietary intervention studies are warranted to establish the proposed efficacy of diets high in protein to improve glycemic control in type 2 diabetes patients.

Though there are ample suggestions that protein and/or amino co-ingestion with each main meal could represent an effective dietary strategy to improve blood glucose homeostasis in type 2 diabetes, future research should also focus on possible side effects of diets high in protein. There has been much debate on the safety of high protein diets as there are suggestions that an increase in dietary protein intake is associated with the development of nephropathy in type 2 diabetes[68]. Although current research does not seem to support these associations, more longer term (>1 y) intervention studies are warranted to assess the proposed relationship between increasing protein intake and the development of renal complications.

Besides addressing the benefits and potential health risks of long-term protein and/or amino acid supplementation in future research, research should continue to elucidate the various parameters that determine the postprandial insulin secretory response to the co-ingestion of protein, protein hydrolysates, and/or free amino acids as most of the underlying mechanisms remain to be elucidated. There are suggestions that besides amino acid composition also protein structure can be of great influence in the subsequent insulinotropic response. Protein hydrolysates have shown to be digested and/or absorbed at a

much faster rate than intact proteins[69, 70] which could augment the insulinotropic properties of the protein by inducing an earlier rise in plasma amino acids concentrations. Furthermore, specific amino acids and amino acid combinations have been shown to have different insulinotropic properties[71-75]. Many of these amino acid specific properties have not yet been determined. Clearly, more research is warranted to define the protein, protein hydrolysate, and/or amino acid mixture with the optimal insulinotropic properties when co-ingested with (mixed) meals.

General conclusions

In this thesis, we investigated whether protein and/or amino acid ingestion can be applied as a dietary interventional strategy to increase postprandial insulin secretion and improve blood glucose homeostasis in type 2 diabetes patients. It is shown that current pharmacological standards of diabetes treatment with oral blood glucose lowering medication are insufficient to obtain the desired level of glycemic control. Postprandial blood glucose excursions are clearly shown to represent a severely underestimated problem in type 2 diabetes patients, as the prevalence of postprandial hyperglycemia is generally not accurately assessed when applying current standard measures of glycemic control (i.e. fasting blood glucose concentration and hemoglobin A1c content).
The blunted insulin secretory response to glucose which is characteristic in longstanding type 2 diabetes patients is not necessarily associated with an impaired insulin secretory capacity of the β-cell. Co-ingestion of a protein hydrolysate with or without additional free amino acids (and in particular leucine) has been proven to be an effective nutritional strategy to further augment glucose stimulated insulin release. The greater insulin secretory response can accelerate postprandial glucose disposal and attenuate the postprandial rise in circulating blood glucose concentrations. Therefore, the blunted insulinotropic response to glucose seems to be related to impairments in glucose sensing and signaling pathways in the pancreatic β-cell. The branched chain amino acid leucine is a compound of special interest as it can stimulate endogenous insulin release and might also augment β-cell mass. Leucine co-ingestion was reported to further augment the insulinotropic properties of protein hydrolysate ingestion with carbohydrate. In accordance, co-ingestion of a protein hydrolysate with additional free leucine with each main meal was shown to reduce the prevalence of hyperglycemia throughout the day under standardized dietary but otherwise free living conditions. Co-ingestion of the same amount of protein hydrolysate without additional leucine with each main meal did not seem to be of sufficient impact to substantially reduce the prevalence of hyperglycemia. Co-ingestion of protein and the accompanying insulin response have been associated with an increase in muscle protein synthesis rate. The latter is of specific clinical relevance to the

type 2 diabetes patient as the gradual loss of muscle mass with aging represents both cause as well as consequence of insulin resistance. We show that the postprandial muscle protein synthetic response to the combined ingestion of protein and carbohydrate does not seem to be severely impaired in type 2 diabetes patients when compared with matched, normoglycemic controls.
This thesis provides ample evidence to show that protein and/or amino acid co-ingestion represents an effective nutritional strategy to stimulate postprandial insulin secretion, accelerate blood glucose disposal and, as such, improve glycemic control in type 2 diabetes patients.

Future research

The research described in this thesis shows that protein, protein hydrolysate and/or amino acid co-ingestion represents an effective nutritional intervention strategy to improve glycemic control in type 2 diabetes patients. However, the performed research also led to many new questions that remain to be addressed. To define an optimal nutritional modulation in which protein ingestion can be implemented in an effective and feasible way to improve glycemic control in type 2 diabetes, we need to address the following topics in future research:

- What protein source has the strongest insulinotropic properties and what are the parameters that define the insulinotropic property of a protein source?
- Does modulation of protein digestion and absorption kinetics change the insulinotropic response when co-ingested with carbohydrate?
- Do high protein diets promote the insulinotropic response to meal ingestion and improve glycemic control?
- Is protein hydrolysate or amino acid co-ingestion the most effective strategy to reduce postprandial hyperglycemia?
- Is there an adaptive insulinotropic response to proteins/amino acids when implemented in long-term intervention studies?
- Does the insulinotropic response to protein/amino acids differ between patients during the progression of the disease?
- What are the long-term effects of diets high in protein? Do high protein diets induce weight loss by stimulating satiety and reducing fat and/or carbohydrate intake?
- Are there any health concerns regarding long-term protein/amino acid co-ingestion in nutritional interventions?
- What are the additional benefits of protein/amino acid co-ingestion within a lifestyle intervention program that combines dietary modulation and increased physical activity on glycemic control in type 2 diabetes patients?

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Summary

Type 2 diabetes is a chronic metabolic disease which currently affects approximately 6% of the world population. Type 2 diabetes is primarily characterized by hyperglycemia which is the result of insulin deficiency or reduced insulin sensitivity. Besides the conventional pharmacological treatment of this disease, lifestyle interventions have shown to be successful in improving glycemic control in this population. Protein and amino acid co-ingestion has been proposed as an effective nutritional strategy to stimulate postprandial insulin secretion, improve glucose disposal and, as such, to improve glycemic control in type 2 diabetes patients. This thesis evaluates the efficacy of such nutritional interventions to improve glucose homeostasis in type 2 diabetes patients.

In chapter 2 we confirm that the insulin response to carbohydrate ingestion is severely impaired in longstanding type 2 diabetes patients. However, the postprandial insulin response following carbohydrate ingestion can be substantially improved by co-ingesting a protein and amino acid mixture. The increase in insulin secretion was shown to improve glucose disposal and attenuate the postprandial rise in blood glucose concentrations in type 2 diabetes patients.

Amino acids are able to stimulate insulin secretion through multiple mechanisms. Most amino acids can be used as a substrate source by the β-cell for ATP production. The greater ATP production increases the ATP/ADP ratio, resulting in the closure of ATP-sensitive K^+ channels, and leading to membrane depolarization. The branched chain amino acid leucine is of particular interest as it has both insulinotropic and metabolic properties. In chapter 3, we tested the insulinotropic potential of protein and/or additional leucine co-ingestion in a more practical setting. We assessed the impact of protein hydrolysate and protein hydrolysate plus leucine co-ingestion with a single meal-like amount of carbohydrate in both type 2 diabetes patients and healthy controls. Co-ingestion of a protein hydrolysate increased the postprandial insulin response significantly. Addition of free leucine resulted in a even greater insulin response in longstanding type 2 diabetes patients. Co-ingestion of protein hydrolysate or protein hydrolysate plus leucine improved the postprandial glucose responses to the same extend.

Postprandial hyperglycemia is the major determinant of 24 h glycemia and represents a direct and independent risk factor for the development of cardiovascular disease in diabetes. As protein hydrolysate co-ingestion can modulate postprandial insulin secretion and augment glucose disposal, we speculated that protein hydrolysate and/or leucine co-ingestion would represent an effective nutritional strategy to improve postprandial glycemic excursions. To determine its impact in a real life setting we applied continuous glucose monitoring in chapters 4, 5 & 6 to investigate glycemic excursions and the possible benefits of protein hydrolysate and leucine co-ingestion on glucose homeostasis under standardized dietary but otherwise free living conditions. In

chapter 4, we assessed 24 h glycemia in healthy subjects and type 2 diabetes patients under standard medical care. Under normal free living conditions, and when consuming a healthy and balanced diet, hyperglycemia was evident less than 40 min during the day in healthy, normoglycemic controls. In contrast, type 2 diabetes patients who continued their normal medication were in a state of hyperglycemia for more than 13 h per day. These results clearly show that the current pharmacological approach is insufficient in the treatment of type 2 diabetes and that postprandial hyperglycemia is a severely underestimated problem in type 2 diabetes. In chapter 5 we determined the impact of protein hydrolysate and leucine co-ingestion on the prevalence of hyperglycemia under such standardized, but otherwise free living conditions. Co-ingestion of protein and additional leucine was shown to substantially reduce the prevalence of hyperglycemic blood glucose excursions by 25%. In chapter 6, we performed the same study without the additional co-ingestion of free leucine. This was of practical relevance as amino acid fortification is currently under debate, and as such, does not yet represent a feasible interventional strategy to improve glycemic control in type 2 diabetes patients. However, in this study we did not detect a measurable reduction in the prevalence of hyperglycemia when co-ingesting a protein hydrolysate with each main meal.

The age-related loss of skeletal muscle mass is a major contributing factor associated with the development and progression of whole-body insulin resistance and type 2 diabetes. Therefore, it is evident that prevention of the age related loss of muscle mass forms an important therapeutic target in type 2 diabetes. As protein ingestion is an important anabolic stimulus contributing to muscle mass maintenance, we aimed to compare the protein anabolic response to protein hydrolysate ingestion between elderly type 2 diabetes patients and healthy control subjects in chapter 7. Protein hydrolysate co-ingestion with carbohydrate was shown to stimulate whole-body protein balance and muscle protein synthesis rates to a similar extend in the diabetes patients and normoglycemic controls.

The research presented in this thesis shows that protein hydrolysate and/or amino acid co-ingestion represent an effective nutritional strategy that can be used to stimulate postprandial insulin secretion and improve glycemic control in type 2 diabetes patients.

Samenvatting

Type 2 diabetes is een chronisch metabole ziekte waaraan momenteel ~6% van de wereldbevolking lijdt. Type 2 diabetes wordt hoofdzakelijk gekenmerkt door een verhoogde bloedglucose concentratie (hyperglycemie) dat het gevolg is van insuline deficiëntie en een verminderde gevoeligheid voor insuline. Naast de conventionele farmacologische behandeling van diabetes zijn aanpassingen in het voedingspatroon en het verhogen van de mate van dagelijkse fysieke activiteit (zogenaamde 'lifestyle' interventies) succesvol gebleken in het verbeteren van de bloedglucose homeostase bij type 2 diabetes patiënten. Er zijn suggesties dat de gecombineerde inname van eiwit (hydrolysaten) en/of aminozuren met de maaltijd een efficiënte voedingsinterventie zou kunnen vormen om de postprandiale insuline productie te stimuleren en de bloedglucose klaring te vergroten. Een verhoogde bloedglucose klaring zou kunnen leiden tot een verbetering van de glucose homeostase van type 2 diabetes patiënten. Dit proefschrift evalueert de mogelijkheden van een dergelijke voedingsinterventie bij type 2 diabetes patiënten.

In hoofdstuk 2 bevestigen wij dat de glucose gestimuleerde insuline secretie ernstig verstoord is in type 2 diabetes patiënten die al langere tijd gediagnostiseerd zijn. De gecombineerde inname van een eiwit/aminozuur mengsel kan de glucose gestimuleerde insuline secretie echter aanzienlijk verbeteren in deze groep patiënten. De verhoogde insuline productie versnelt de glucose klaring met als gevolg een minder sterke stijging van de bloedglucose concentratie na koolhydraat consumptie. Er zijn verschillende mechanismen verantwoordelijk voor de aminozuur geïnduceerde insuline productie. De meeste aminozuren kunnen fungeren als substraatbron voor de productie van ATP in de β-cel. Door de toename in ATP productie vindt er een stijging plaats van de ATP:ADP ratio waardoor K^+ kanalen zich sluiten. Dit leidt tot membraan-depolarisatie wat resulteert in insuline excretie. Het vertakte keten aminozuur leucine heeft bijzondere insulinotrope en metabole eigenschappen en geniet om deze reden extra aandacht. In hoofdstuk 3 werd de insulinotrope respons op de consumptie van eiwit met én zonder extra leucine getest in een meer praktische situatie. Hierbij werd de insuline respons op de gecombineerde inname van een eiwithydrolysaat of een eiwithydrolysaat/leucine mengsel getest in combinatie met een hoeveelheid koolhydraten zoals die ook in een normale maaltijd voorkomt. De gecombineerde inname van een eiwithydrolysaat verhoogde de postprandiale insuline secretie aanzienlijk. De toevoeging van vrij leucine leidde echter tot een nog sterkere stijging in insuline secretie in type 2 diabetes patiënten. De verhoogde postprandiale insuline secretie ging gepaard met een significante verlaging van de bloedglucose respons.

Postprandiale hyperglycemie is de belangrijkste determinant van 24-uurs glycemie en vormt een directe en onafhankelijke risicofactor voor de ontwikkeling van cardiovasculaire aandoeningen bij type 2 diabetes patiënten. Aangezien de gecombineerde inname van een eiwithydrolysaat en

koolhydraten de postprandiale insuline productie verder stimuleert en de glucoseklaring verhoogd, speculeerden wij dat de gecombineerde inname van een eiwithydrolysaat plus vrij leucine bij iedere maaltijd een effectieve voedingsinterventie zou kunnen vormen om de postprandiale bloedglucose homeostase te verbeteren. Om deze hypothese in een praktische situatie te toetsen hebben wij in de hoofdstukken 4, 5 & 6 gebruik gemaakt van continue glucose meters. In hoofdstuk 4, werden deze meters gebruikt om bloedglucose concentraties gedurende 24 uur te bepalen in gezonde deelnemers en type 2 diabetes patiënten die orale bloedglucose verlagende medicatie gebruiken. Onder normale levensomstandigheden en met een gezonde, evenwichtige voeding was er bij de gezonde normoglycemische personen slechts sprake van hyperglycemie gedurende 40 min per dag. De type 2 diabetes patiënten daarentegen verkeerden meer dan 13 uur in een staat van hyperglycemie ondanks de continuering van hun medicatiegebruik. Deze resultaten laten duidelijk zien dat de huidige farmacotherapeutische benadering in de behandeling van type 2 diabetes ontoereikend is. Het is dus duidelijk dat postprandiale hyperglycemie een ernstig onderschat probleem vormt bij type 2 diabetes patiënten. In hoofdstuk 5 werd de invloed onderzocht van eiwithydrolysaat en leucine supplementatie op de prevalentie van hyperglycemie onder normale levensomstandigheden. De supplementatie van eiwit plus leucine direct na iedere maaltijd reduceerde de prevalentie van hyperglycemische bloedglucose excursies met ruim 25%. Aangezien de toevoeging van vrije aminozuren aan voedingsmiddelen momenteel niet is toegestaan werd in hoofdstuk 6 hetzelfde experiment herhaald maar dan zonder de toevoeging van extra leucine. Echter, in deze studie kon geen meetbare reductie van de prevalentie van hyperglycemie aangetoond worden.
Veroudering is geassocieerd met een verlies van skeletspiermassa en dit verlies is een belangrijke factor die bijdraagt aan de ontwikkeling van insuline resistentie en type 2 diabetes. Om deze reden zou de preventie van leeftijdsgerelateerd spiermassaverlies een belangrijk therapeutisch doel moeten vormen bij de preventie en behandeling van type 2 diabetes. Het doel van hoofdstuk 7 was daarom om te onderzoeken of de eiwitsynthese na eiwitinname even sterk gestimuleerd kan worden in type 2 diabetes patiënten als in gezonde normoglycemische controle personen. De gecombineerde inname van koolhydraten met een eiwithydrolysaat resulteerde in een aanzienlijke stijging van de spiereiwitsynthese, maar bleek niet verschillend tussen type 2 diabetes patiënten en gezonde controles.
Op basis van de resultaten beschreven in dit proefschrift kan geconcludeerd worden dat de gecombineerde inname van een insulinotroop eiwithydrolysaat/aminozuur mengsel een effectieve voedingsinterventie vormt om de postprandiale insuline secretie te bevorderen en daarmee de bloedglucose homeostase te verbeteren in type 2 diabetes patiënten.

Dankwoord

Ondanks dat míjn naam op de voorkant van dit boekje staat zou ik dit niet hebben bereikt zonder de hulp van velen die, al dan niet op wetenschappelijke wijze, hebben geholpen in de totstandkoming van dit proefschrift.

Als eerste wil ik mijn promotieteam bestaande uit Luc van Loon, als co-promotor en dagelijks begeleider, en Wim Saris en Ton Wagenmakers, mijn promotoren, bedanken. Luc, ik ben ontzettend blij dat ik bij jou terecht ben gekomen en dat jij mijn "baas" bent geworden. Jij hebt mij de kans gegeven om dingen te bereiken die ik jaren geleden niet voor mogelijk had geacht. Ondanks dat je het steeds drukker krijgt sta je toch altijd voor iedereen klaar. Behalve dat we samenwerken zie ik je ook als vriend. Ik denk niet dat er veel AIO's zijn die met hun begeleider naar muziekconcerten gaan, alleen jammer dat we soms in de alcoholvrije zone terecht kwamen. Ton, helaas hebben wij niet zoveel kunnen samenwerken als we misschien hadden gewild door jouw vertrek naar Birmingham. Desondanks was je altijd beschikbaar om weer eens een artikel te lezen of om na te denken over de resultaten van een studie. Wim, jij bent eigenlijk altijd een stabiele factor geweest, meestal enigszins op de achtergrond maar niet te min aanwezig met je gedachten en raad, bedankt hiervoor.

Next, I would like to thank all the members of the review committee Prof. Arie Nieuwenhuijzen Kruseman, Prof. Marleen v. Baak, Prof. Philip Newsholme, Prof. Annemie Schols & Prof. Bruce Wolffenbuttel for their time and their critical assessment of my dissertation. Daarnaast wil ik ook Prof. Ellen Blaak en Dr. Arie Kies hartelijk bedanken voor het feit dat jullie plaats willen nemen in de corona.

Ook ben ik veel dank verschuldigd aan mijn paranimfen, Erik en Maartje. Erik, wij kennen elkaar al sinds het begin van onze studietijd in Maastricht en het was eigenlijk vanaf het begin van mijn AIO-schap vanzelfsprekend dat jij mijn paranimf zou worden. Jouw scherpe humor heeft al bij menig kamergenoot *("ik bel ivm de witte tijger"*) tot verwarring geleid en heeft zelfs een van onze secretaresses het schaamrood op de kaken bezorgd. Nú ben ik aan de beurt maar over een paar maanden is het tijd voor jouw verdediging en ik vind het een eer dat ik dan achter jou mag staan. Maartje, wij kennen elkaar nog niet zo lang, en het klikte ook niet van begin af aan tussen ons maar dat is gelukkig helemaal goed gekomen. Zo zie je maar, de eerste indruk is niet altijd de juiste. Ik heb zeer veel waardering voor jou en voor wat je doet. Daarom vind ik het ook erg jammer dat we geen kamergenoten meer zijn, maar gelukkig hebben we de foto's nog.

In den beginnen waren er drie, maar in de loop van de tijd is onze M^3-groep onder leiding van Luc aanzienlijk gegroeid. René, wij hebben jaren zeer intensief samengewerkt wat ook wel blijkt uit het feit dat jij co-auteur bent op

verschillende van mijn publicaties. Ook al waren de testdagen vaak erg lang toch heb ik die altijd erg leuk gevonden. We hadden veel lol en we hadden aan een half (of soms zelfs geen) woord genoeg als een van de pompen weer kuren had of als een van de proefpersonen zijn infuus er weer eens had uitgetrokken. Jouw nuchterheid was een welkome afwisseling en ook onze "data" besprekingen heb ik altijd zeer op waarde geschat. Ik vind het jammer dat jij er vandaag niet bij kan zijn maar Melbourne is nu eenmaal niet om de hoek. Antoine, Milou, Lex, Joan, Dominique, Stephan, Hanneke, Richard, Bart, Marika, Suzanne, Ben, Trent, Jodil, Maurice & Kristof, bedankt voor de fijne samenwerking de afgelopen jaren, en hopelijk gaat dat nog wel een tijdje door. In dit dankwoord mag ik natuurlijk ook niet vergeten om Milou (2e keer), Ruth (blijven streven!!), Margit, Bart G., Sandra, Tamara, Cindy, Annemiek en Lotte te bedanken die mij als stagiaires hebben geholpen bij de uitvoering van mijn studies en mij veel werk uit handen hebben genomen.

Door de jaren heen heb ik verschillende kamergenoten gehad, enkele daarvan heb ik al genoemd maar de rest mag ik ook zeker niet vergeten. Niels, Alie, Johan, Michael, Denis en Eric bedankt voor de leuke tijd en de goede gesprekken. Voordat ik begin met alle (oud)collega's en vrienden te bedanken eerst speciale aandacht voor de spil van HB, het secretariaat. Claudia, Larissa, Ilona, Desirée (voor al het geregel de laatste tijd) en Kirsten, bedankt dat jullie altijd klaarstonden, en staan, om te helpen. Jos en Wendy S., bedankt voor alle analytische ondersteuning en Gabby en Macro bedankt voor de praktische ondersteuning aan het begin van mijn AIO-tijd. Anneke, Astrid, Andrea, Boris, Cheryl, Chantal, Desirée B., (b)Elke, Esther P., Esther M., Eefje, Ellen, Freek, Floortje, Gijs (trainingsbeest), Guy, Gert, Geert, Harm, Herman, Hasibe, Janneke, Joost O., Joris, Julia & Marie-Jeanne (de twee Dames van de twee Heeren), Judith, Kaatje, Kristel, Karen, Kathleen, Katharina, Loek, Lydia, Mandy, Matthijs, Marja, Miranda, Petra, Paul, Ronnie, Sander, Sandy, Silvie, Stefan, Stijn, Uriël, Vera & Wendy B. bedankt voor alles binnen en vooral ook buiten de Uni. Ook veel dank aan de "Dames van het Sirc" en de "Jongens van de spoelkeuken" voor al jullie hulp en ondersteuning door de jaren heen.

Alle proefpersonen die deelgenomen hebben aan mijn studies hartelijk bedankt en sorry voor alle vieze drankjes die ik jullie heb voorgeschoteld. Zonder jullie was dit proefschrift er niet geweest. Ik heb veel van jullie geleerd op zowel het wetenschappelijke als het persoonlijke vlak.

Luc, Cindy, Joris, Roy, Arie, André en Marian van DSM Food Specialties, bedankt voor de fijne samenwerking en de vele discussies die we in Maastricht en Delft hebben gevoerd. Hanneke v. Milligen van Menarini Diagnostics, bedankt voor je hulp bij het opzetten van het GCM systeem in Maastricht en natuurlijk ook voor alle dropjes (mede namens Luc) en de gezellige etentjes.

De vrienden van het eerste uur, Anita, Janneke, Robin en Susanne maar ook Steven, Wout en Michiel ben ik veel dank verschuldigd. Jullie waren er gedurende de afgelopen jaren altijd, voor de leuke dingen van het leven maar ook voor die tijden dat ik last had van de AIO-blues.

Dan rest mij nu alleen nog maar twee mensen te bedanken, Riny en Jan.
Mam, wij hebben altijd al een heel speciale band gehad en ik hou heel veel van je. We hebben het alles behalve gemakkelijk gehad maar we waren er altijd voor elkaar en dat zal ook altijd zo blijven. Jan, ook al ben je het niet, je bent van begin af aan als een vader voor mij geweest en ik hoop dat jullie met zijn tweetjes nog vele gelukkige jaren voor de boeg hebben.

Curriculum vitae

Ralph Manders was born on August 13 1978 in Venray, the Netherlands. He attended secondary school at the Scholengemeenschap Stevensbeek in Stevensbeek where he graduated from the MAVO, HAVO and VWO. In 1998 he started his study Health Sciences at Maastricht University. He obtained his Masters degree in 2003 in the field of Movement Sciences after an internship with Dr. Hageman and Dr. Hesselink at the department of Farmacology & Toxicology at Maastricht University. During this time he also worked as a research assistant at de department of Internal Medicine developing methods to determine inflammatory responses in the human digestive tract.
In February 2003, he started as a PhD student at the department of Human Biology at Maastricht University. In 2006, he was awarded a 2nd place young investigator award by the Netherlands Organisation of Scientific Research for his work on nutritional interventions to obtain glycemic control in type 2 diabetes patients.
Currently Ralph Manders is working as a Post Doctoral fellow at the department of Human Movement Sciences at Maastricht University where he continues his work studying the effects of nutrition and exercise on glycemic control in type 2 diabetes patients through the application of continuous glucose monitoring.

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